Discovery of Novel Adenosine Receptor Agonists that Exhibit Subtype Selectivity

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Running Title: Investigating adenosine receptor selectivity of N^6-modified agonists.

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Abstract

A series of N^6-bicyclic and N^6-(2-hydroxy)cyclopentyl derivatives of adenosine were synthesized as novel A_1R agonists and their A_1R/A_2R selectivity assessed using a simple yeast screening platform. We observed that the most selective, high potency ligands were achieved through N^6-adamantyl substitution in combination with 5'-*N*-ethylcarboxamido or 5'-hydroxymethyl groups. In addition, we determined that 5'-(2-fluoro)thiophenyl derivatives all failed to generate a signaling response despite showing an interaction with the A_1R. Some selected compounds were also tested on A_1R and A_3R in mammalian cells revealing that four of them are entirely A_1R-selective agonists. By using *in silico* homology modeling and ligand docking, we provide insight into their mechanisms of recognition and activation of the A_1R. We believe that given the broad tissue distribution, but contrasting signaling profiles, of adenosine receptor subtypes these compounds might have therapeutic potential.

Introduction

Adenosine receptors (ARs) belong to the family of G protein-coupled receptors (GPCRs) and exist as four different subtypes, A_1, A_{2A}, A_{2B} and A_3. All subtypes respond to the purinergic nucleoside adenosine, but they have a wide and varying tissue distribution. Many ARs have been linked to cardiovascular, respiratory and inflammatory disorders.[1] Furthermore, in the central nervous system they have been implicated in acute pathological conditions such as epilepsy, hypoxia and ischemia,[2,3] and chronic neurodegenerative disorders, such as Parkinson's, Alzheimer's and Huntington's diseases.[4] In human cells, the A_1R and A_3R predominantly couple to the $G\alpha_i$ family of G proteins, inhibiting the production of cAMP, while the A_2R subtypes couple to the $G\alpha_s$ subunit, stimulating adenylate cyclase to elevate cAMP levels. Given their common ligands, diametrically opposed effects and overlapping tissue distribution the ARs have been the focus of extensive research to discover subtype selective ligands. However, limitations of mammalian systems can hinder the testing and development of these compounds. For instance, the A_1R can signal through the $G\alpha_{i1}$, $G\alpha_{i3}$ and $G\alpha_o$[5] but it is currently difficult to differentiate between these effectors in an *in vivo* mammalian cell-based assay.

Most of the known AR agonists are based on the adenosine scaffold and receptor subtype selectivity can be achieved by substituting the purine ring of the nucleoside at positions C-2 and/or N^6 with appropriate functional groups. For instance, substitution of the N^6-position with bulky cycloalkyl- and bicycloalkyl groups has resulted in A_1R-selective agonists.[6-10] Introduction of a wide range of N^6-substituents is conveniently achieved by nucleophilic aromatic substitution of the corresponding 6-chloro purine precursor with primary or secondary amines. The ribose moiety, in particular at the C-2', C-3' and C-5' positions, have also been the subject of many modifications which can influence A_1R affinity, selectivity and efficacy.[8,11,12] 5'-Carboxamido adenosine derivatives, such as the prototypical AR agonist 5'-*N*-ethylcarboxamidoadenosine (NECA), among many other examples, are known to be potent activators. More bulky groups, such as substituted 5'-thioaryl and 5'-oxoaryl moieties have also been explored and these studies have provided novel A_1R-selective and potent agonists.[13,14] In light of this, we designed a

series of adenosine analogues that feature different cyclic and bicyclic substituents at the N^6 of the purine ring and various functional groups at the C-5' of the ribose in order to assess the effect of these modifications on AR activity and subtype selectivity.

We[15-20] and others[5,21-23] have previously described the use of modified *Saccharomyces cerevisiae* strains containing chimeric (yeast-human) G protein alpha subunits to functionally couple heterologously expressed GPCRs. Specifically, the chimeric G proteins enable mammalian GPCRs to functionally couple to the yeast-mating pathway. This pathway includes a reporter (*FUS1-lacZ*) gene providing a quantitative assay for GPCR activation.[16] The yeast platform provides a simple, affordable and robust assay with which to identify novel GPCR ligands and their interactions with a single effector.[17,24,25] This system has also been established to study A_1R, $A_{2A}R$ and $A_{2B}R$ in a number of G protein backgrounds,[5,18,21-23] although evidence of functional couplings of the A_3R has not been reported.

In this study we exploit the yeast system to characterize novel synthetic adenosine derivatives for their agonist activity against the A_1R, $A_{2A}R$ and $A_{2B}R$. We explored subtype selectivity further at the A_1R and A_3R in mammalian CHO-K1 cells for the compounds that were active against the A_1R in the yeast screen. Moreover, we use homology modeling and docking to gain insight into the binding of our agonists at the A_1R. Our yeast-based screen and mammalian cell assays have identified novel adenosine nucleosides exhibiting interesting A_1R selective profiles. Hence, they constitute valuable tool compounds for cellular studies and might have therapeutic potential.

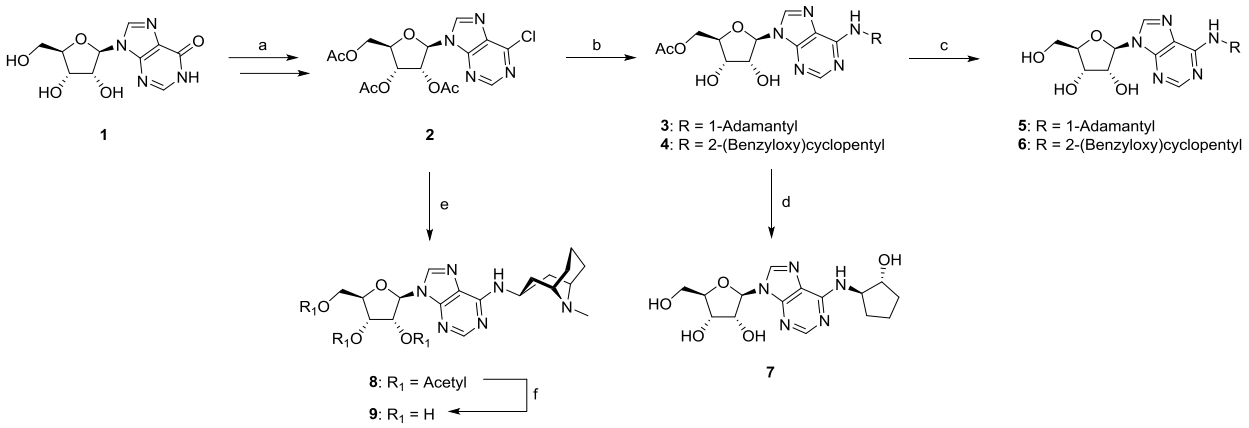
Results and Discussion

Chemistry

Known compounds **5**,[26] **6**[27] and **7**[28] have previously been shown to be selective for the A_1R with respect to their binding affinity. These analogues were prepared for assessment using our yeast-based assay. Bulky bicyclic groups have been highlighted as beneficial for A_1R selectivity,[7-10] so we also prepared novel

compound **9**. Intermediate **2** was required for efficient generation of the analogues (Scheme 1). This was synthesized according to the procedure adopted by Kotra *et al.*,[29] with minor experimental modifications.

Scheme 1. Synthesis of Adenosine Derivatives[a]



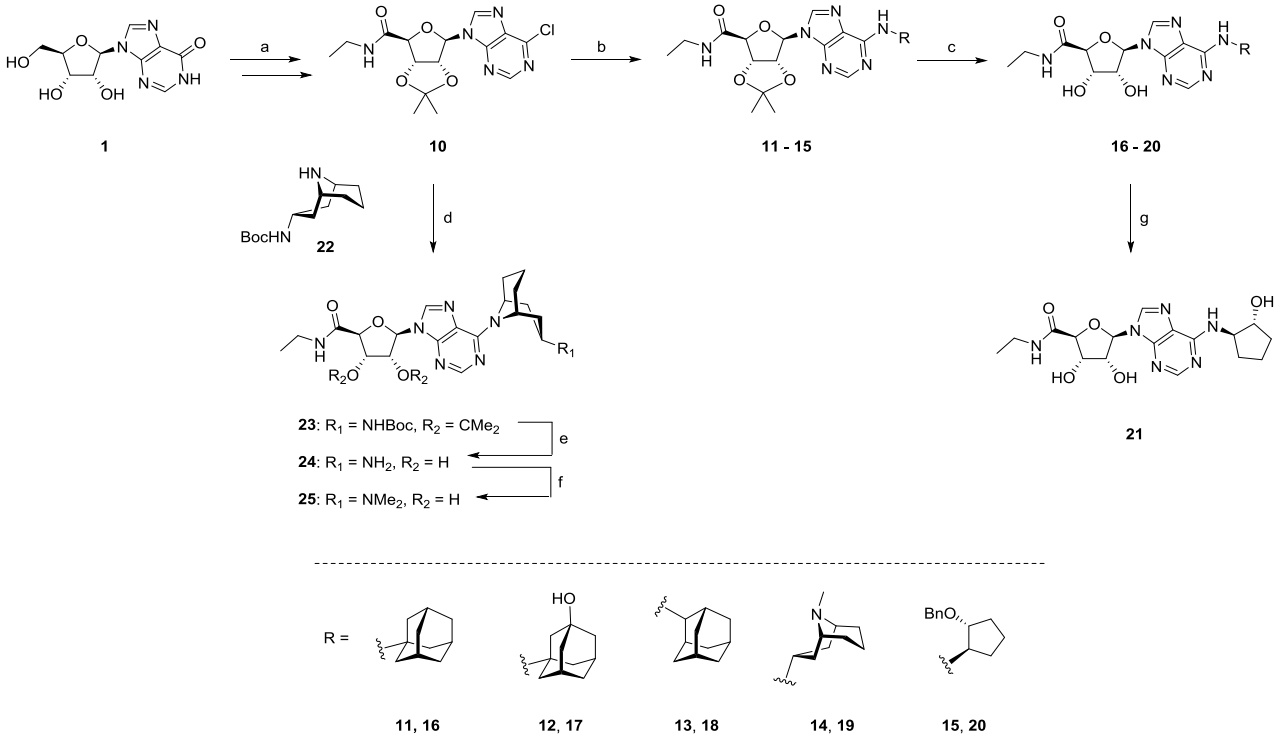
[a] Reagents and conditions: (a) procedures according to reference 29; (b) R-NH_2, Et_3N or DIPEA, EtOH, reflux, 18 h to 5 days (for specific conditions and yields see *Experimental Section*); (c) K_2CO_3, MeOH, rt, 3 h, 99%; (d) **4**, $Pd(OH)_2$, cyclohexene, ethanol, reflux, 18 h, 99%; (e) (1*R*,3*r*,5*S*)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine, DIPEA, EtOH, reflux, 18 h, 54%; (f) K_2CO_3, MeOH, rt, 3 h, 99%.

Aromatic substitution of the N^6-chloro group with 1-adamantylamine or (1*R*,2*R*)-1-amino-2-benzyloxycyclopentane was carried out in the presence of triethylamine or Hünig's base. This resulted in partial deacetylation to give the monoacetylated products (**3** and **4**), which could be attributed to the use of excess base. The presence of the acetyl on the primary alcohol was confirmed using ^{1}H NMR and was then removed using potassium carbonate in methanol to give **5** and **6** in quantitative yield. The choice of base did not appear to have an effect on the reaction. Attempts to directly remove the benzyl group from **6** using hydrogenolysis returned unreacted starting material. However, treatment of monoprotected **4** with Pearlman's catalyst and cyclohexene afforded **7**. Interestingly, aromatic substitution with (1*R*,3*r*,5*S*)-9-

methyl-9-azabicyclo[3.3.1]nonan-3-amine (granatanamine) using less base did not result in deacetylation and afforded the expected product **8**. Deprotection was again carried out with potassium carbonate and methanol. Granatanamine was prepared according to a procedure previously developed in our group.[30]

Despite the widespread use of 5'-*N*-ethylcarboxamidoadenosine (NECA) as an A_1R agonist we found that the analogous cyclopentyl (**21**) and adamantyl (**16**) congeners were novel compounds and to the best of our knowledge untested at the A_1R. This is possibly a consequence of the non-selective nature of NECA at the AR subtypes.[31] In this case intermediate **10** was required to allow generation of novel analogues (Scheme 2).

Scheme 2. Synthesis of Novel NECA Derivatives[a]



[a] Reagents and conditions: (a) procedures according to reference 32; (b) R-NH_2, Et_3N or DIPEA, EtOH, reflux, 18 h to 5 days (for specific conditions and yields see *Experimental Section*); (c) acetic acid, water, 80 °C, 18 h, 99%; (d) *tert*-butyl-9-azabicyclo[3.3.1]nonan-3-yl carbamate (**22**), DIPEA, EtOH, reflux, 18

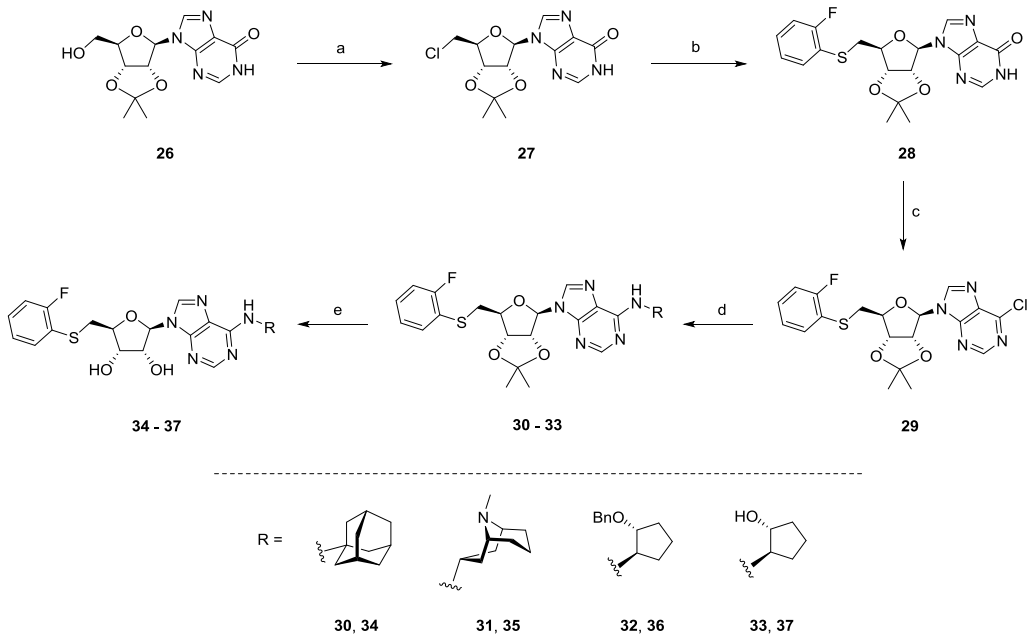
h, 85%; (e) acetic acid, water, 80 °C, 18 h, 99%; (f) formic acid, formaldehyde (37% aq.), reflux, 18 h, 63%; (g) **20**, $Pd(OH)_2$, cyclohexene, ethanol, reflux, 18 h, 99%.

The amide building block **10** was prepared from **1** as previously reported by Middleton *et al*..[32] Displacement of the chloride in **10** with the appropriate amine in the presence of either Hünig's base or triethylamine gave intermediates **11–15**. This reaction proceeded with ease in refluxing ethanol overnight in the case of **13–15**, however all 1-adamantyl analogues required one week at reflux to generate sufficient quantities of desired compounds. Interestingly, the 2-adamantylamine reaction was complete within one day. Acetonide deprotection was achieved by heating overnight in acetic acid and water to generate **16–20** with quantitative yields observed. Removal of the benzyl protecting group from **20** with palladium hydroxide and cyclohexene to generate **21** proceeded in quantitative yield. We decided to prepare analogues **24** and **25** with the alternative bicyclic architecture to allow us to probe the necessity for a secondary amine at the adenine N^6 position. Chloride **10** was reacted with granatyl secondary amine **22**, which was prepared according to a literature protocol.[33] Like the adamantyl analogues, this reaction was very slow and required reflux for one week to generate sufficient quantities of product to give **23**. Concomitant deprotection of the acetonide and Boc group occurred on treatment with acetic acid and water to give **24**. The dimethylamine **25** was then prepared using formic acid and formaldehyde.

Given the prior studies on CVT-3619 (**37**) showing that it is a specific partial agonist at the A_1R[14] we prepared this compound for assessment using our yeast-based assay and planned to prepare new analogues with the 2-fluorothiophenol group at the C-5' position of the ribose ring. In alignment with our strategy for adenosine and NECA analogues we required chloride **27**, which would allow efficient generation of analogues with various cyclic groups at the N^6 position of the adenine (Scheme 3). Primary chloride **27** was prepared from protected **26** using Appel conditions according to the previously reported procedure.[34] Treatment with 2-fluorothiophenol and sodium hydride gave **28** in 48% yield and subsequent chlorination with our previously adopted conditions of thionyl chloride and DMF gave **29** in 88% yield.

Introduction of the cyclic component was accomplished by reacting with the appropriate primary amine in the presence of DIPEA or triethylamine to give **30**–**33**. Again, preparation of the adamantyl analogue required one week at reflux to obtain the product in sufficient yield. Initial attempts to directly remove the benzyl protecting group from **32** with palladium hydroxide and cyclohexene to generate **37** were unsuccessful. However, 6-chloropurine **29** reacted readily with (1*R*,2*R*)-2-aminocyclopentanol to generate the desired product directly. Acetonide deprotection with acetic acid and water at reflux generated final compounds **34**–**37** in excellent yields.

Scheme 3. Synthesis of CVT-3619 and New Derivatives[a]



[a] Reagents and conditions: (a) CCl_4, PPh_3, DMF, rt, 18 h, 53%; (b) 2-fluorothiophenol, NaH, DMF, 0 °C to rt, 3 h, then chloride **27**, DMF, rt, 18 h, 48%; (c) $SOCl_2$, DMF, DCM, 50 °C, 5 h, 88%; (d) R-NH_2, Et_3N or DIPEA, EtOH, reflux, 18 h to 3 days (for specific conditions and yields see *Experimental Section*); (e) acetic acid, water, 80 °C, 18 h, 99%.

Biological Activity

We expressed all four AR subtypes, under the control of the constitutive *GAPDH* promoter, in a panel of transplant yeast strains engineered to contain chimeric Gα-subunits in which the 5 C-terminal amino acids of Gpa1p have been replaced with those mammalian $G\alpha_q$, $G\alpha_{12}$, $G\alpha_o$, $G\alpha_{i1/2}$, $G\alpha_{i3}$, $G\alpha_z$ and $G\alpha_s$. Efficient trafficking of the A_1R, $A_{2A}R$ and $A_{2B}R$ to the cell surface in yeast cells was confirmed using modified receptors engineered to contain a GFP fluorophore at the C-terminus (Figure 1A). NECA is a non-subtype selective AR agonist and was used to determine through which Gα-subunits each receptor signaled. Yeast cells were exposed to 100 µM NECA for 16 hours and reporter gene activity (as measured through β-galactosidase production) was determined (Figure 1B-D).

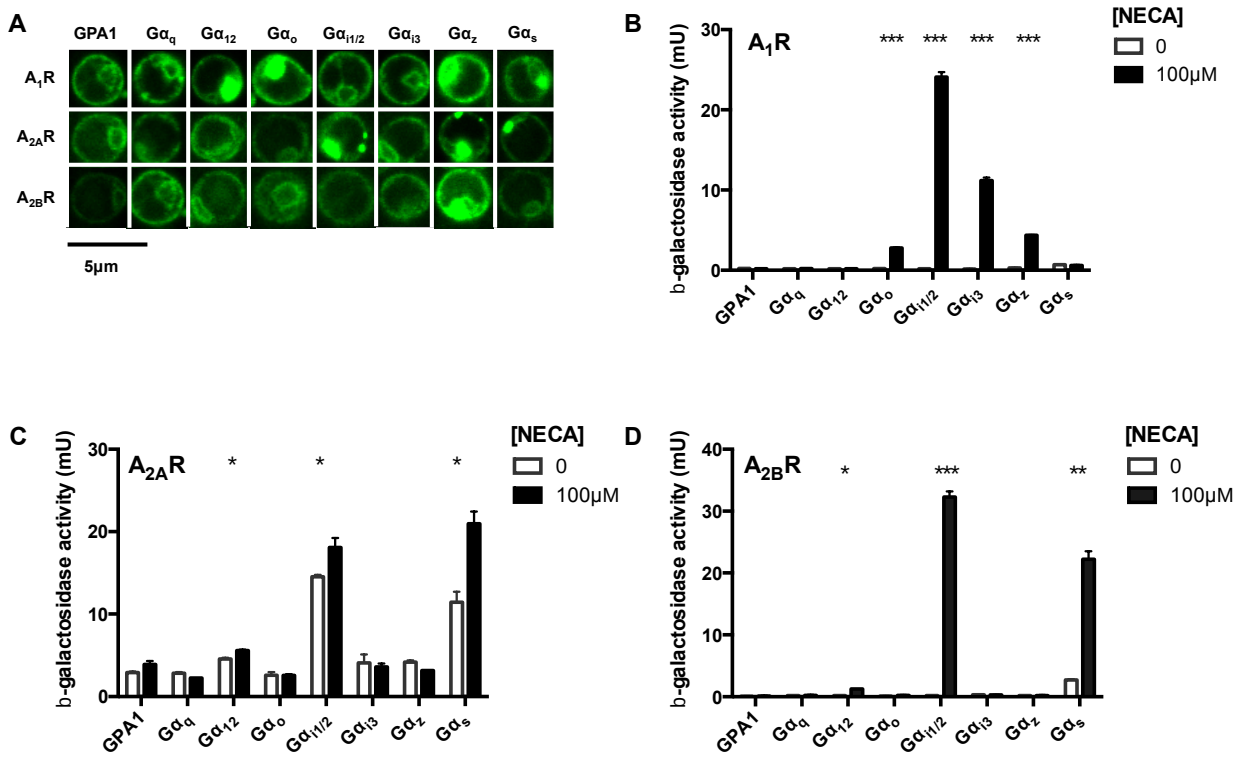


Figure 1. NECA-activated yeast-mating pathway via specific AR/Gα protein chimeras. (A) A C-terminal GFP tag was engineered onto the A_1R, $A_{2A}R$ and $A_{2B}R$ and expression at the plasma membrane was confirmed using fluorescence microscopy. Scale bar = 5 µm. (B-D) Yeast strains expressing the human (B)

A_1R, (C) $A_{2A}R$ (D) $A_{2B}R$ were stimulated with 0 or 100 μM NECA for 16 h and assayed for the activation of the *FUS1-lacZ* reporter gene as previously described.[15-17,19] β-galactosidase units (mU) are expressed as the ratio of *o*-nitrophenol product to cell density (determined colorimetrically; see *Experimental Section*). Data are the mean of 5 independent experiments ± SEM. Data were determined as significantly different from the non-ligand response using Student's *t*-test where *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Consistent with previous reports[5,22] the A_1R generated significant ($p < 0.05$) responses in strains expressing G protein chimeras corresponding to $G\alpha_o$, $G\alpha_{i1}$ and $G\alpha_{i3}$ (Figure 1B). In addition, we also report for the first time, functional coupling of the A_1R signaling through the GPA1/$G\alpha_z$ transplant. Signaling was not observed via GPA1/$G\alpha_q$, GPA1/$G\alpha_{12}$ or GPA1/$G\alpha_s$ or the unmodified Gpa1p ($n \geq 16$ isolates screened for functionality). Further, we observed that the $A_{2A}R$ and $A_{2B}R$ (Figure 1C and 1D) signaled through both GPA1/$G\alpha_s$ and GPA1/$G\alpha_{i1}$ but we failed to identify any functional coupling for the A_3R in our panel of strains (Supplementary Figure S1). Moreover, we report that, the $A_{2A}R$ displayed significantly elevated levels of ligand-independent signaling which is consistent with previous observations in yeast.[18] While we have observed that these GPCRs can couple to a number of different Gpa1p chimeras, we have chosen to focus on the ones that are widely reported to be the most physiologically relevant in mammalian cells. Consequently, the A_1R-GPA1/$G\alpha_{i1}$, $A_{2A}R$-GPA1/$G\alpha_s$ and $A_{2B}R$-GPA1/$G\alpha_s$ strains were chosen for further compound characterization.

Subtype Selectivity of Adenosine Derivatives in Yeast. Having identified yeast strains that functionally express the A_1R, $A_{2A}R$ and $A_{2B}R$, we sought to validate their pharmacology in response to a range of agonists. Dose-response curves were determined for NECA, adenosine, 2-chloro-N^6-cyclopentyladenosine (CCPA) and CGS-21680 (Figure 2) using the yeast reporter assay. Sigmoidal dose-response curves were observed allowing the maximum response (E_{max}) and potency (pEC_{50}) to be determined (Table 1). NECA, adenosine and CCPA are full agonists at the A_1R (E_{max} compared with NECA by one-way ANOVA, $p > 0.05$) but have differing potencies (Table 1). This generates a rank order of

potency for the ligands of CCPA > NECA > adenosine. CGS-21680 had a much lower potency than the other ligands (pEC_{50} = 3.2 ± 0.1) and failed to reach a maximal response. While the overall potency values are lower than observed in mammalian cells, the rank order of the ligands is conserved between yeast and mammalian systems.[35] Application of the operational model of pharmacological agonism[36] enabled calculation of the ligand binding affinity (pK_A) and efficacy (τ) (Table 2). In comparison to NECA, both adenosine and CCPA have a greater pK_A and a reduced τ.

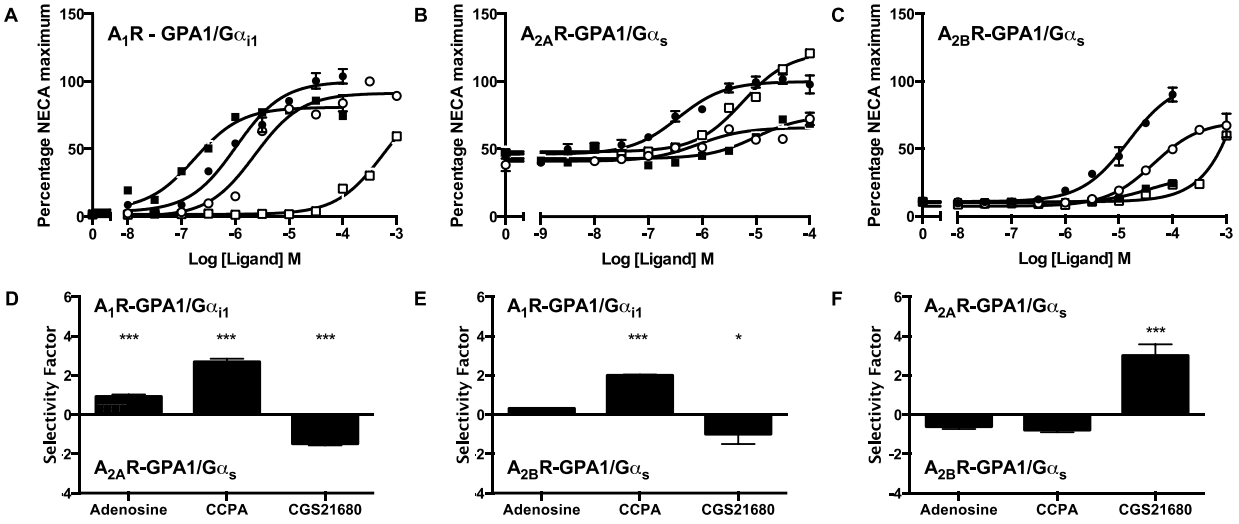
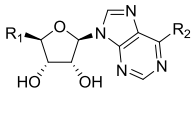
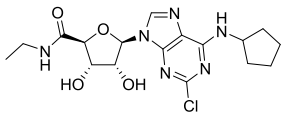


Figure 2. AR agonists display receptor subtype selectivity. Dose-response curves for various AR agonists were generated from yeast strains expressing (A) A_1R, (B) $A_{2A}R$ and (C) $A_{2B}R$ following stimulation for 16 h with (●) NECA, (○) adenosine, (■) CCPA or (□) CGS-21680. Activation of the reporter gene was calculated and is expressed as the percentage of the maximum response achieved when cells were stimulated with the reference agonist NECA. (D-F) Receptor selectivity was calculated as the change in log (τ/K_A), relative to NECA, for the data in A-C. Data were determined as statistically different (**, $p < 0.01$, ***, $p < 0.001$) from NECA, using a one-way ANOVA with Bonferroni's post-test. All data are mean of 5-8 independent experiments ± SEM.

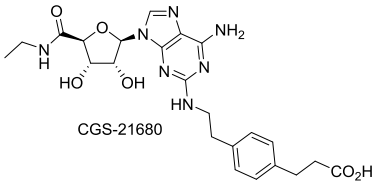
Table 1. Potency (pEC_{50}) and Maximal Response (E_{max}) of Reference Compounds and Synthetic Adenosine Derivatives at A_1R, $A_{2A}R$ and $A_{2B}R$ as measured in Yeast[a]



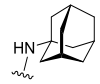
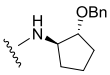
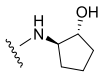
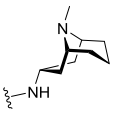
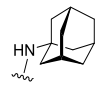
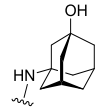
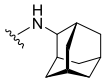
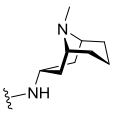
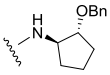
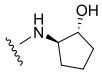
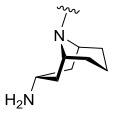
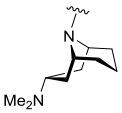
Adenosine, NECA, **5-7**, **9**, **16-21**, **24**, **25**, **34-37**

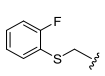
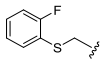
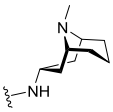
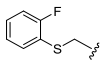
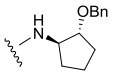
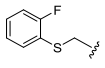
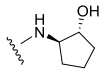


CCPA



CGS-21680

Compd	R_1	R_2	A_1R		$A_{2A}R$		$A_{2B}R$	
			pEC_{50}[b]	E_{max}[c]	pEC_{50}[b]	E_{max}[c]	pEC_{50}[b]	E_{max}[c]
NECA	-CONHEt	$-NH_2$	6.0 ± 0.1	100 ± 2.4	6.47 ± 0.2	100 ± 2.0*	4.83 ± 0.1	100 ± 3.8
Adenosine	$-CH_2OH$	$-NH_2$	5.6 ± 0.1*	91.4 ± 2.8	5.24 ± 0.8	65.9 ± 1.7**	4.29 ± 0.1	70.4 ± 2.9**
CCPA	-	-	6.7 ± 0.1***	80.9 ± 2.2	5.00 ± 0.2	74.7 ± 4.9	4.14 ± 0.1	30.6 ± 2.0***
CGS-21680	-	-	3.2 ± 0.1***	91.3 ± 14.1	4.70 ± 0.1*	121.7 ± 4.1	2.20 ± 0.6***	59.1 ± 2.8***
5	$-CH_2OH$		4.8 ± 0.1***	98.5 ± 4.1	N.R.[d]		N.R.	
6	$-CH_2OH$		5.8 ± 0.1	89.8 ± 1.8	N.R.		N.R.	
7	$-CH_2OH$		6.2 ± 0.1	83.7 ± 4.4	N.R.		N.R.	
9	$-CH_2OH$		N.D.[e]		N.R.		N.R.	
16	-CONHEt		5.4 ± 0.0***	104.4 ± 1.3	N.R.		N.R.	
17	-CONHEt		4.0 ± 0.1***	100 ± 23.7	N.R.		N.R.	
18	-CONHEt		4.8 ± 0.0***	107.7 ± 3.0	N.R.		N.R.	
19	-CONHEt		N.D.		N.R.		N.R.	
20	-CONHEt		6.0 ± 0.1	99.3 ± 0.3	4.8 ± 0.3*	115.0 ± 13.0	3.9 ± 0.3	32.2 ± 11.4***
21	-CONHEt		6.5 ± 0.1**	99.0 ± 2.8	5.24 ± 0.3	77.7 ± 4.6	3.48 ± 0.3*	49.2 ± 0.4***
24	-CONHEt		N.R.		N.R.		N.R.	
25	-CONHEt		N.R.		N.R.		N.R.	

34			N.R.	N.D.	N.R.
35			N.R.	N.R.	N.R.
36			N.R.	N.R.	N.R.
37			N.R.	N.R.	N.R.

Values are the mean ± SEM from 5-8 independent repeats.

[a] A_1R and A_2R receptors in GPA1/$G\alpha_{i1}$ and GPA1/$G\alpha_s$ yeast transplants, respectively.

[b] Negative logarithm of the agonist concentration required to induce a half-maximal response.

[c] The maximal response to a ligand expressed as a percentage of that obtained for NECA.

[d] N.R., no response.

[e] N.D., not determined. Full dose-response curve was not feasible.

Statistical significance compared to NECA (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

Table 2. Ligand Affinity (pK_A) and Efficacy (log τ) of Reference Compounds and Synthetic Adenosine Derivatives at A_1R, $A_{2A}R$ and $A_{2B}R$ expressed in Yeast[a]

Compd	A_1R		$A_{2A}R$		$A_{2B}R$	
	pK_A[b]	log τ[c]	pK_A[b]	log τ[c]	pK_A[b]	log τ[c]
NECA	4.4 ± 0.1	1.5 ± 0.1	5.9 ± 0.2	0.5 ± 0.1	4.2 ± 0.1	0.6 ± 0.1
Adenosine	4.6 ± 0.2	0.9 ± 0.1	5.6 ± 0.2	0.0 ± 0.3	3.8 ± 0.1	0.3 ± 0.1
CCPA	6.1 ± 0.1	0.6 ± 0.1	4.7 ± 0.3	-0.1 ± 0.1	4.4 ± 0.1	3.5 ± 0.1***
CGS-21680	2.1 ± 0.9*	1.1 ± 0.9	4.9 ± 0.1	-0.3 ± 0.0	3.4 ± 0.1	-0.2 ± 0.0
5	3.0 ± 1.1	1.8 ± 1.1	N.R.[d]		N.R.	
6	4.9 ± 0.1	0.5 ± 0.1	N.R.		N.R.	
7	5.5 ± 0.1	0.7 ± 0.1	N.R.		N.R.	
9	N.R.		N.R.		N.R.	
16	4.2 ± 0.3	1.2 ± 0.3	N.R.		N.R.	
17	3.5 ± 0.1	1.0 ± 0.1	N.R.		N.R.	
18	2.4 ± 0.1*	2.4 ± 0.0	N.R.		N.R.	
19	N.R.		N.R.		N.R.	
20	4.3 ± 0.5	1.6 ± 0.5	3.9 ± 0.7**	0.5 ± 0.5	3.5 ± 0.6	0.1 ± 0.5
21	4.8 ± 0.5	1.6 ± 0.5	4.6 ± 0.4	0.3 ± 0.1	4.2 ± 0.1	-0.2 ± 0.1
24	N.R.		N.R.		N.R.	
25	N.R.		N.R.		N.R.	
34	N.R.		N.D.[e]		N.R.	
35	N.R.		N.R.		N.R.	
36	N.R.		N.R.		N.R.	
37	N.R.		N.R.		N.R.	

Values are the mean ± SEM from 5-8 independent repeats.

[a] A_1R and A_2R in GPA1/$G\alpha_{i1}$ and GPA1/$G\alpha_s$ yeast transplants, respectively.

[b] Negative logarithm of the relative equilibrium disassociation constant for each compound generated through use of the operational model of agonism.[36]

[c] The coupling efficiency parameter (τ), generated by comparison to NECA.

[d] N.R., no response.

[e] N.D., not determined. Full dose-response curve was not feasible.

Statistical significance compared to NECA (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

We next sought to investigate the pharmacological properties of the $A_{2A}R$ expressed in our GPA1/$G\alpha_s$ expressing strains. Both CGS-21680 and NECA displayed strong agonism at the $A_{2A}R$, while adenosine and CCPA showed weak partial agonism (Figure 2B). Further, all ligands assayed display weak potency at the $A_{2B}R$, (rank ligand potencies, NECA > adenosine = CCPA >> CGS-21680) with CGS-21680 failing to generate a maximal response at the ligand concentrations assayed. Thus, in our strains, CGS-21680 would appear to be largely $A_{2A}R$ selective and this is consistent with mammalian cell affinity data.[31,35]

We next sought to compare the selectivity/preference that ligands may possess for each of the ARs. Expression of the ARs in yeast generates a clean, robust assay, with no competing signaling machinery, so enabling the proportioning of receptor responses to individual signaling pathways. We have previously used the methods developed by Figuero *et al.*[37] to quantify ligand bias for receptors expressed in yeast.[19,20] Here we report the adaptation of the equimolar method of comparison[37] to quantify a ligands selectivity for a given receptor (see *Experimental Section* for more details). Since NECA is a full agonist for all three ARs expressed in yeast, it can be used as a reference ligand. By calculating the change in log (τ/K_A), for an agonist relative to NECA, for each AR subtype we have generated a quantitative means of comparing receptor selectivity for all our agonists (Figure 2D-F). Adenosine and CCPA are A_1R-selective but also preferentially activate $A_{2B}R$ over $A_{2A}R$. In contrast, CGS-21680 is A_2R subtype-selective with an overall preference for the $A_{2A}R$.

Bulkier N^6-adamantyl agonists have previously been shown to be A_1R selective with respect to binding affinity at rat receptors.[26] Therefore we extended our studies to include novel AR agonists containing an adamantyl group. **5**, **16-18** were derived from adenosine and NECA respectively (Schemes 1-2). These compounds appeared to be A_1R selective full agonists compared with NECA, with no significant response detected at the $A_{2A}R$ and $A_{2B}R$ ($p > 0.05$) (Figure 3A-C). However, **5**, **16-18** displayed reduced potency to the A_1R compared to their precursors and cyclopentyl variants (Table 1). Furthermore, pK_A values suggested this might be a consequence of reduced ligand binding affinities (Table 2).

A_1R agonists derived from adenosine with substituted cyclopentyl groups at the adenine N^6 position have been reported previously (e.g. GR79236, *N*-[(1*S*,2*S*)-(2-hydroxy)cyclopentyl]adenosine).[38] We found that N^6-cyclopentyl derivatives **6**, **7**, **20** and **21** mainly signal through the A_1R, but for the latter two compounds minimal responses were also detected at $A_{2A}R$ and $A_{2B}R$ (Table 1, Figure 4).

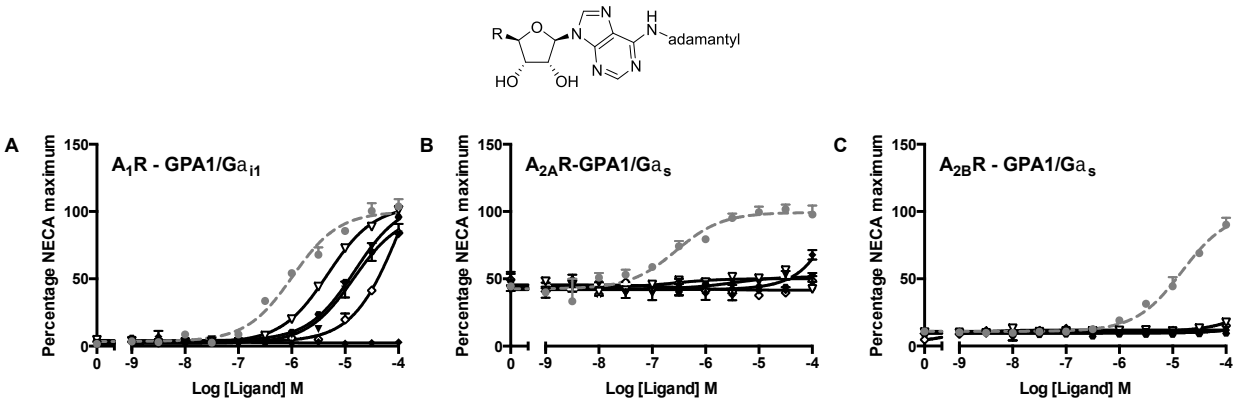


Figure 3. N^6-adamantyl derivatives selectivity at the human A_1R. Yeast strains expressing (A) A_1R, (B) $A_{2A}R$ and (C) $A_{2B}R$ were stimulated with N^6-adamantyl derivatives (▼) **5**, (▽) **16**, (◇) **17**, (●) **18** and (◆) **34** for 16 h and reporter gene activity determined. Data are expressed as the percentage of the maximum response achieved when cells were stimulated with the reference agonist NECA (grey dotted line). All data are mean of 5-8 independent experiments ± SEM.

Adenosine derivative **6** was highly A_1R selective but failed to produce a maximal signal. In a patent in 2011[27] **6** was described as an A_1R-selective agonist for reducing elevated intraocular pressure in the treatment of glaucoma or ocular hypertension and this compound was assessed for selectivity with respect to binding affinity (K_i) at human subtypes A_1, A_{2A} and A_3.[27] In accordance with our results it was shown, using a radioligand displacement assay, that **6** binds with greater than 250-fold affinity at the A_1 over the A_{2A} subtype. In the yeast-based assays the novel NECA analogues **20** and **21** have equal or higher potency, respectively, than the parent compound NECA at the A_1R. However, in contrast to the N^6-cyclopentyl adenosine derivatives **6** and **7**, N^6-cyclopentyl NECA derivatives **20** and **21** are non-selective and signal

through the A_1R, $A_{2A}R$ and $A_{2B}R$ (Table 1). Calculation of selectivity factors confirm that **21** preferentially signals $A_1R > A_{2B}R > A_{2A}R$.

As described above, the N^6-adamantyl derivatives only induced a detectable response in A_1R-expressing yeast strains, suggesting that bulky N^6-substituents promote total A_1R/A_2R selectivity. To explore this further we created a series of ligands containing an N^6-azabicyclo (granatane) moiety. Compounds **9**, **19**, **24** and **25** were screened for activity; however, no significant response was detected for these compounds via the A_1R, $A_{2A}R$ or $A_{2B}R$ ($p > 0.05$, one-way ANOVA). Some minimal response was observed for **9** and **19** at very high concentration (100 μM) but it was not feasible to generate full dose-response curves (Table 1).

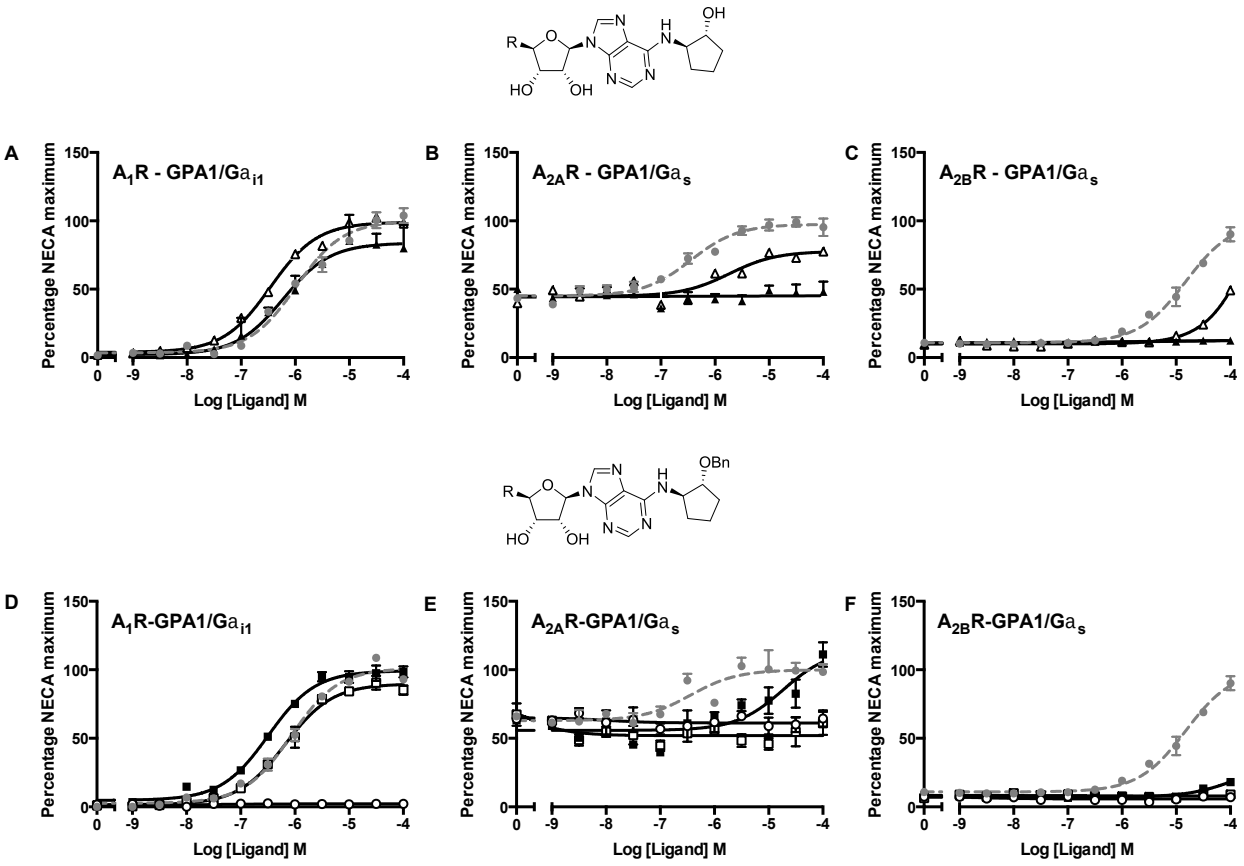


Figure 4. N^6-(2-Hydroxy)cyclopentyl derivatives of both adenosine and NECA display bias towards the A_1 receptor. Yeast strains expressing the A_1R (A and D), the $A_{2A}R$ (B and E) and the $A_{2B}R$ (C and F) were stimulated for 16 h with N^6-(2-hydroxy)cyclopentyl derivatives (▲) **7** and (△) **21** or N^6-(2-

benzyloxy)cyclopentyl derivatives (□) **6**, (■) **20** and (○) **36** and assayed for activation of the *FUS1*> *lacZ* reporter gene. Data are expressed as the percentage of the maximum response achieved when cells were stimulated with the reference agonist NECA (grey dotted line). Data are mean of at least five independent experiments ± SEM.

Replacement of the 5'-ethyl carboxamide or 5'-hydroxy group with a 2-fluorothiophenyl moiety, as in adenosine analogues **34**-**37**, resulted in compounds that failed to produce any detectable response in the A_1R, $A_{2A}R$ and $A_{2B}R$ strains (Table 1). In fact, **34** was only able to activate the $A_{2A}R$ but at concentrations of greater than 100 μM. These results were somewhat surprising since the N^6-hydroxycyclopentyl congener **37** (CVT-3619, later named GS 9667) has previously been described as a selective, partial agonist of the A_1R, with reported K_i values of 113 nM and 1.1 μM when challenged with the antagonist [^{3}H]CPX, binding in hA_1R-expressing DDT_1MF-2 and CHO cells, respectively.[14] To determine if the lack of functional activity for **34**, **36** and **37** in our yeast assays resulted from the compounds failing to cross the membrane, we performed a competition assay between **34**, **36** and **37** and either NECA, adenosine or CCPA at the A_1R (Supplementary Figure S2).

In line with the previously reported data,[14] we confirmed **37** does appear to compete with all three ligands at the A_1R ($pA_2 = 5.3 \pm 0.4$). Interestingly, despite the fact that **37** interacts with the A_1R, in our experimental system, it appears unable to induce a measurable response. It is worth noting that, A_1R agonist activity of **37** (CVT-3619) was previously demonstrated in rat adipocytes where it reduced cAMP content and consequently lipolysis[14] although it is entirely possible that the observed response in these cell lines resulted from "off-target activation" of other receptors. Despite entering clinical trials where it was evaluated for its efficacy to lower lipids and thus improve glycemia, CVT-3619 (**37**) showed inadequate pharmacokinetics and it was discontinued.[39] Furthermore, some recent studies suggest that the A_1R may not play a significant role in hepatic regulation of lipid metabolism.[40] Similar to **37**, close analog **36** also

acted as a competitive antagonist at A_1R ($pA_2 = 6.4 \pm 0.2$, Supplementary Figure S2) but **34** did not appear to bind to the A_1R at all.

Determining A_1R versus A_3R Selectivity in Mammalian Cells. Traditionally, many compounds that display selectivity for the A_1R compared to the A_2Rs frequently also display activity to the A_3R. However, as described previously, we were unable to obtain functional coupling of the A_3R to the yeast pheromone-response pathway (Supplementary Figure S1). Thus, to provide a complete characterization of the A_1R-selective compounds isolated in the yeast screen, we utilized mammalian CHO-K1 cells transiently transfected with either the A_1R or the A_3R. CHO-K1 are an established cell line frequently used to assay the activity of adenosine receptors.[41,42] Both the A_1R and the A_3R couple to the inhibitory G protein family ($G\alpha_i$) thereby reducing the cellular concentration of cAMP.

Using CHO-A_1R cells (CHO-K1 cells expressing the A_1R) we first confirmed that NECA, adenosine and CCPA were able to inhibit forskolin-stimulated cAMP production (Figure 5A) generating pIC_{50} values (Table 3) equivalent to those previously reported.[41,42] Further, all compounds (**5**, **6**, **7**, **16**, **17**, **18, 20** and **21)** identified in the yeast as eliciting a functional A_1R response displayed full agonist activity against the A_1R in the mammalian cells, but with varying potencies (Figure 5B, Table 3). Significantly, when these compounds were assayed against the CHO-A_3R cells (Figure 5C and 5D, Table 3) only **7**, **17**, **20** and **21** were able to inhibit forskolin-stimulated cAMP production. Thus, taken together these data suggest that, at concentrations $\leq 1\mu M$, **5**, **6**, **16** and **18** display A_1R-selectivity. Intriguingly, 2-adamantyl derivative **18** is almost as potent an agonist of A_1R as NECA but completely A_1R-selective.

Table 3. Potency (pIC_{50}) and response range of reference ligands and putative A_1R selective compounds at the A_1R and A_3R as measured in transfected CHO-K1 cells.

Compd	A_1R			A_3R		
	pIC_{50}[a]	Response Range[b]	n	pIC_{50}[a]	Response Range[b]	n
NECA	9.68±0.16	-73.4±5.8	6	9.31±0.17	-44.1±3.0	8
Adenosine	8.63±0.11*	-66.4±3.7	6	8.94±0.14	-38.7±2.4	5
CCPA	9.30±0.15	-71.4±4.3	6	7.95±0.13**	-46.6±4.8	8
5	7.72±0.20**	-61.0±6.1	5	N.R	N.R	8
6	9.17±0.15	-55.28±3.6	4	N.R.	N.R.	6
7	8.36±0.17*	-60.0±4.4*	6	7.56±0.11**	-35.1±2.2	5
16	8.54±0.3*	-52.15±6.0*	6	N.R	N.R.	6
17	7.43±0.19	-39.3±3.9**	4	7.1±0.12**	-32.7±2.2*	6
18	9.40±0.34	-51.07±7.9	6	N.R	N.R	6
20	10.53±0.28*	-48.35±5.6*	4	6.57±0.15***	-38.0±4.0	5
21	8.21±0.2*	-47.67±4.3*	4	8.64±0.08	-37.9±1.2	5

Data are the mean ± SEM of *n* individual sets.

[a]The negative logarithm of the agonist concentration required to produce a half-maximal response.

[b]The response range of the agonists expressed as a percentage of total forskolin range (0-100%).

Statistical significance compared to NECA (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

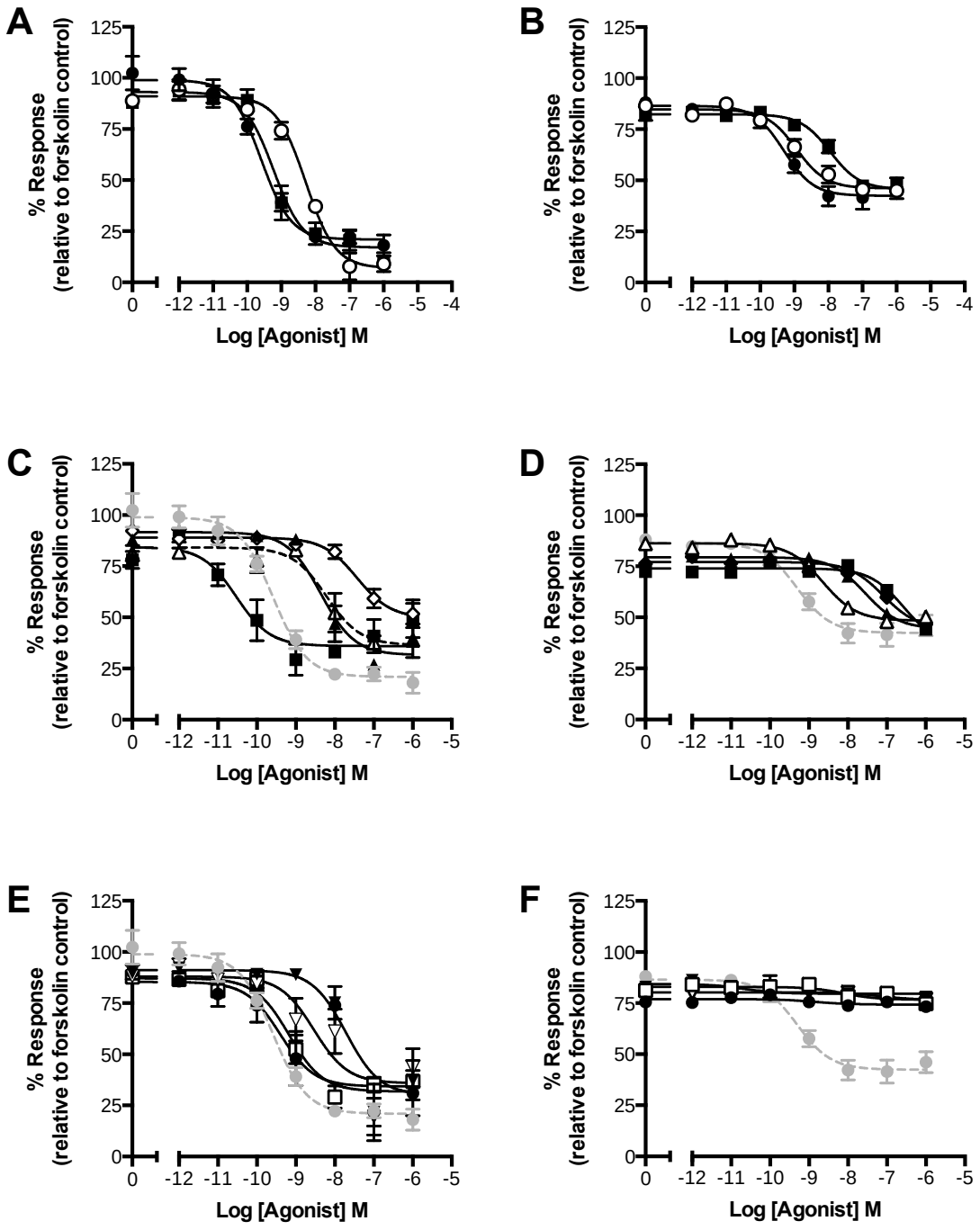


Figure 5. Determining the A_1R-selectivity of compounds isolated in the yeast screen against the A_1R and A_3R expressed in mammalian cells. CHO-K1 cells transiently transfected with A_1R (A, C and E) or A_3R (B, D and F) were stimulated with (●) NECA, (○) adenosine, (■) CCPA (A and B) or the compounds determined to be active at the A_1R from the yeast screen (▲) **7**, (◇) **17**, (■) **20,** (△) **21** (C and D) (▼) **5**,

(□) **6**, (▽) **16,** (●) **18** (E and F). In panels (C-F) NECA dose-inhibition curve is shown as grey dashed line with grey symbols. All cells were assayed for inhibition of 10 μM forskolin-stimulated cAMP. Data are expressed as the percentage of the maximum response achieved when cells were stimulated with 10 μM forskolin. Data are mean of 4-8 independent repeats ± SEM.

Molecular simulation of agonist docking into the A_1R

Based on the pharmacological experimental findings that most of our N^6-substituted 5'-*N*-ethylcarboxamido and 5'-hydroxymethyl derivatives activated the A_1R, but all the 5'-(2-fluoro)thiophenyl derivatives failed to do so, we used a molecular modeling approach to dock all our synthetic adenosine derivatives into a homology model of the human A_1R. The recently solved crystal structure of the human A_{2A}R complexed with the agonist UK-432097 (PDB ID: 3QAK)[43] served as the template for this homology model. We reasoned that the bound agonist UK-432097 has a large N^6-substituent (Figure 6A) as is the case for our synthetic adenosine analogues.

In order to validate the utility of this A_{2A}R crystal structure as a template for generating the A_1R homology model we docked compounds **20**, **21** and **34**, that also showed activity at the A_{2A}R in our assays, into the A_{2A}R crystal structure. Indeed, the proposed positions and side chain interactions for these compounds (Figure 6C and Supplementary Figure S3A and S3B) are very similar to known agonists bound to the A_{2A}R (Figure 6A and 6B).

Furthermore, after closely inspecting the crystal structures of the adenosine-, NECA- (Figure 6B) and UK-432097-bound (Figure 6A) human A_{2A}R (PDB IDs: 2YDO, 2YDV and 3QAK, respectively)[43,44] we would argue that hydrogen bond formation between the ligand and the homologous Thr-91$^{3.36}$, Asn-254$^{6.55}$, Thr-277$^{7.42}$ and His-278$^{7.43}$ (superscript: Ballesteros-Weinstein numbering[45]) in the A_1R is important to stabilize the active conformation of the receptor.[43,44] These residues are highly conserved across the AR family. Taking this into consideration (details see *Experimental Section*) the docking yielded ligand

orientations for **5-7**, **16-18**, **20** and **21** (Figure 6D and Supplementary Figure S3C-I) that closely resemble the orientations of UK-432097- and NECA-bound to the human $A_{2A}R$ (Figure 6A and 6B). In this distinct position the ribose moiety binds deeply into the binding pocket potentially forming hydrogen bonds with Thr-91$^{3.36}$, Asn-184$^{5.42}$, Thr-277$^{7.42}$ and His-278$^{7.43}$. The purine ring π-stacks against Phe-171^{ECL2} and can form hydrogen bonds with Asn-254$^{6.55}$, whereas the bulky N^6-substitutes are located near the exit of the binding pocket. We found experimentally that all these compounds were agonists at the A_1R in our yeast-based functional assay. Intriguingly, **6** and **20** were docked before they were tested, and based on our model, predicted to be active agonists, which indeed was the case.

The predicted binding positions for granatane derivatives **9** and **19** are somewhat similar, however, the purine ring is further away from Asn-254$^{6.55}$ so that no hydrogen bonds are suggested (Figure 6E and Supplementary Figure S3J). At very high concentration, **9** and **19** were able to partially activate the A_1R, but it was experimentally not possible to obtain full dose response curves.

Docking of both inactive compounds **24** and **34** yielded binding positions where the purine ring adopts a *syn*-conformation with respect to the ribose (Figure 6F and Supplementary Figure S3K). It is also noticeable that **24** is not in close contact with Trp-247$^{6.48}$, a residue which is highly conserved across the AR family and in a recent molecular dynamics simulation was shown to act as a conformational toggle switch in the receptor activation mechanism.[46] The docking simulations with adenosine derivatives **25**, **35-37**, all of which failed to activate the A_1R, did not return any binding positions for our model.

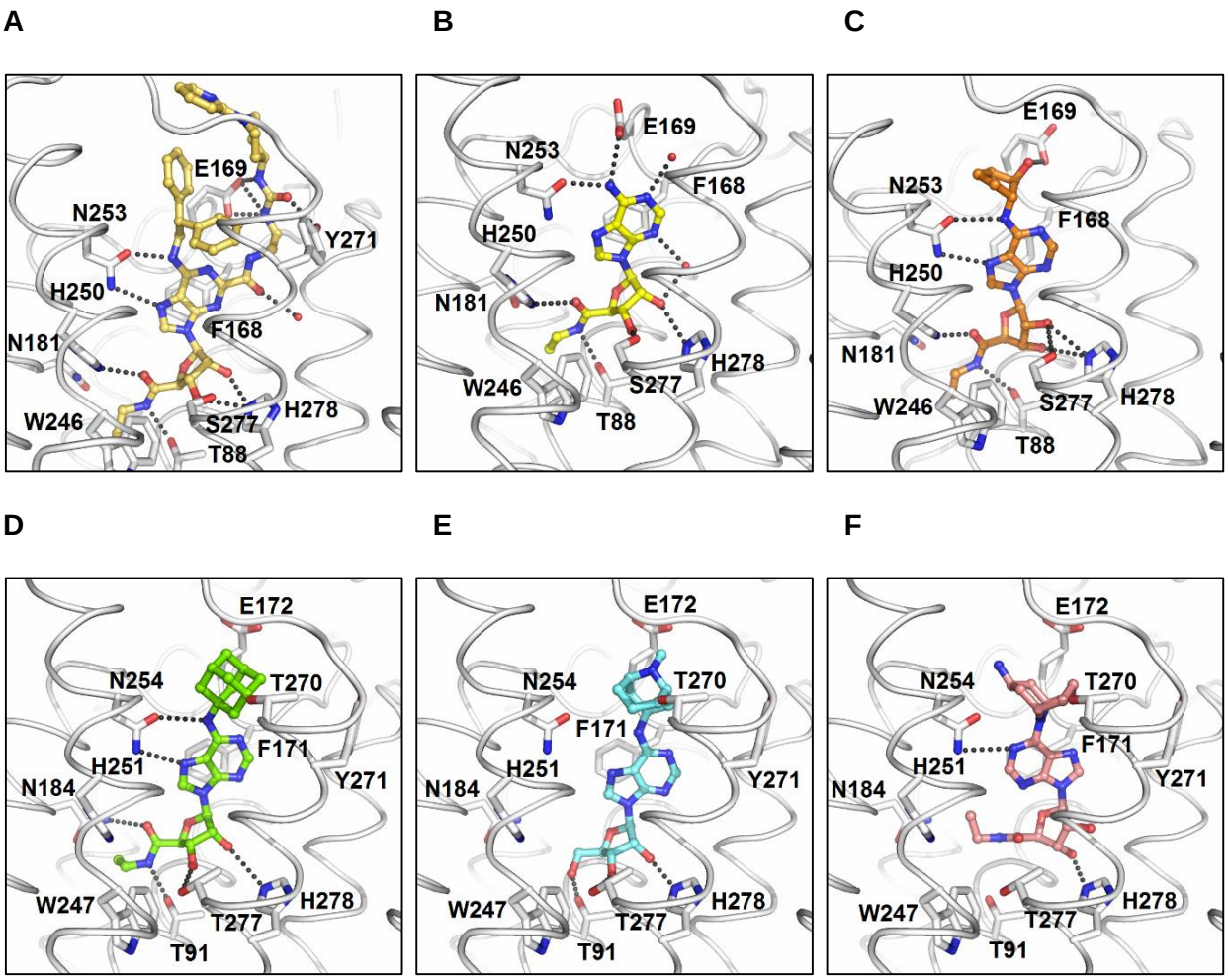


Figure 6. Docking of N^6-substituted adenosine derivatives into an A_1R homology model. (A) Crystal structures of human $A_{2A}R$ bound with agonist UK-432097 (gold, PDB ID: 3QAK) and (B) with agonist NECA (yellow, PDB ID: 2YDV) for comparison, indication key binding residues and interactions. Representative examples of proposed binding poses of N^6-substituted adenosine derivatives in the $A_{2A}R$ crystal structure and in the A_1R homology model. (C) **21** (orange) docked into $A_{2A}R$ crystal structure. (D) **16** (light green), (E) **9** (light blue) and (F) **24** (pink) docked into A_1R homology model. Black dotted lines represent potential hydrogen bonds. Numbering of residues in (A-C) according to P29274 ($hA_{2A}R$) and of homologous residues in (D-F) according to P30542 (hA_1R). Ballesteros-Weinstein (BW) numbering: T88 (A_{2A}), T91 (A_1): BW 3.36; F168 (A_{2A}), F171 (A_1): BW ECL2; E169 (A_{2A}), E172 (A_1): BW ECL2; N181 (A_{2A}), N184 (A_1): BW 5.42; W246 (A_{2A}), W247 (A_1): BW 6.48; H250 (A_{2A}), H251 (A_1): BW 6.52; N253

(A_{2A}), N254 (A_1): BW 6.55; T270 (A_1): BW 7.35; Y271 (A_{2A}), Y271 (A_1): BW 7.36; S277 (A_{2A}), T277 (A_1): BW 7.42; H278 (A_{2A}), H278 (A_1): BW 7.43. For activities of docked compounds see Table 1. Proposed binding poses for the remaining compounds are shown in Figure S3 (*Supporting Information*).

Conclusions

Herein, we report the synthesis of a series of adenosine derivatives that were modified at the N^6-position of the purine ring and the C-5' positions of the ribose moiety. These compounds were evaluated using a yeast-based and mammalian cell-based assay for quantifying their AR subtype selectivity. Our biological data show that compounds bearing a granatane azabicyclic moiety at N^6 and/or a 5'-(2-fluorothiophenyl) substituent at the ribose fail to produce responses in A_1R, $A_{2A}R$ or $A_{2B}R$ cells. Conversely, N^6-adamantyl adenosine and NECA congeners were completely A_1R selective. Moreover, it emerged that N^6-(2-hydroxy)cyclopentyl and N^6-(2-benzyloxy)cyclopentyl derivatives are potent agonists, preferentially activating A_1R over the other subtypes. It is worth noting that novel NECA derivative **20** exhibited higher potency and A_1R selectivity than its parent compound. Further, we present an A_1R homology model that corroborates our experimental findings. Notably, adenosine derivatives **5** and **6**, and novel NECA analogs **16** and **18** are completely A_1R-selective, potent agonists. Therefore they should represent useful tool compounds in purinergic signaling research and warrants further assessment of their therapeutic potential.

Experimental Section

General Chemistry. All reactions were performed under an inert argon atmosphere. Anhydrous tetrahydrofuran (THF), toluene and dichloromethane (DCM) were obtained by filtration through a system of alumina columns under a positive pressure of argon. Anhydrous dimethylformamide (DMF) was purchased as dry over molecular sieves from Sigma-Aldrich. Solvents were evaporated under reduced

pressure at approximately 45 ºC using a Buchi Rotavapor or under high vacuum on a schlenk line. Reagents were purchased from Sigma-Aldrich, Acros, Alfa Aesar, Fischer Scientific or Hänseler and used without further purification. Reactions were monitored by thin layer chromatography (TLC) using aluminium sheets pre-coated with silica (Macherey-Nagel ALUGRAM Xtra SII, G/UV_{254}). Detection was under UV light source (λ_{max} 254 nm) or through staining with potassium permanganate solution (5%), vanillin spray or ninhydrin, with subsequent heating. Flash column chromatography was carried out using silica gel from Sigma-Aldrich (pore size 60Å, 230–400 mesh particle size) as the stationary phase.

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded using a Bruker Avance 300 or an Avance II 400 spectrometer. Chemical shifts (δ_H) are reported in parts per million (ppm) and are referenced to the residual solvent peak. The order of citation in parentheses is (1) number of equivalent nuclei (by integration), (2) multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet) *etc*), (3) coupling constants (*J*) in Hertz (Hz) and (4) assignment. Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded using a Bruker Avance 300 or an Avance II 400 spectrometer. Chemical shifts are quoted in parts per million (ppm) and are referenced to the residual solvent peak. The assignment is quoted in parentheses. COSY, HSQC and DEPT were routinely used to assign peaks in ^{1}H and ^{13}C NMR spectra. Addition of D_2O was used to confirm the assignment of OH and NH peaks. Mass spectra and high resolution mass spectra (HRMS) were recorded on a ThermoScientific LTQ Orbitrap XL spectrometer consisting of a linear ion trap (LTQ) featuring a HCD collision cell, coupled to the Orbitrap mass analyzer, equipped with a nanoelectrospray ion source (NSI). MS and HRMS spectra were determined by the Mass Spectrometry Group at the Department of Chemistry and Biochemistry, University of Bern (PD Dr. S. Schürch).

The purity of the compounds was determined with UPLC-MS on a *Dionex* Ultimate 3000 using a reversed-phase column *Dionex* Acclain RSLC, 120C18, 3 x 50 mm, 2.2 µm, 120Å pore size, flow 1.2 mL/min. The gradient used was 100%A to 100%D over 7 min, with A (water with 0.1% TFA) and D (10%

H_2O/90% ACN+0.1% TFA). Purity was determined by total absorbance at 254 nm. All tested compounds were ≥95% pure, except **17** which was 94% pure.

Established Adenosine Agonists. 5'-*N*-ethylcarboxamidoadenosine (NECA), 2-chloro-N^6-cyclopentyladenosine (CCPA), CGS-21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and SLV-320 were purchased from R & D Systems (Bristol, UK). Where possible, compounds were prepared as 10mM stocks in DMSO.

Chemical Synthesis. Intermediates **2**,[29] **10**,[32] **26**,[32] and **27**[34] were synthesised as described in the literature with only slight experimental modifications. (3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]nonan-3-amine was synthesised as described previously by our group.[30] *tert*-Butyl-9-azabicyclo[3.3.1]nonan-3-ylcarbamate (**22**) was synthesised as described previously.[33] Intermediate **29**[47] is known but was obtained with different methodology. Intermediate **28** is a novel compound. Further details on the synthesis and characterisation of these intermediates are available in the *Supporting Information*. 3-Amino-1-adamantanol and (1*R*,2*R*)-2-aminocyclopentanol hydrochloride were prepared as described in the *Supporting Information*.

General Procedure A for the Synthesis of Intermediates 3, 4, 8, 11–15, 23, 30–33. The appropriate chloride was dissolved in ethanol (20 mL/mmol). The amine and trimethylamine or DIPEA were then added and the reaction mixture was refluxed until TLC analysis indicated completion. The solvent was removed *in vacuo* and the resultant material was purified with column chromatography. The choice of base did not appear to have an effect on the yield or reaction time. In the case of compounds **4**, **8**, **13–15** and **31–33** the reaction was complete after 18 h, whereas sterically hindered **3**, **11**, **12**, **23** and **30** required 5 days refluxing at 120 °C to obtain sufficient amounts of product. In cases where the amine is a hydrochloride salt this was first stirred for 20 min with the base, before addition of the appropriate chloride in ethanol. For specific purification conditions see individual compounds.

General Procedure B for the Synthesis of Compounds 16–20, 24, 34–37. Acetonide protected compounds **11–15**, **23** or **30–33** were dissolved in water and acetic acid and stirred at 80 °C overnight. The water was removed *in vacuo* and the resultant crude material was purified with column chromatography.

General Procedure C for the Synthesis of Compounds 7 and 21. Benzyl protected compounds **4** and **20** were dissolved in ethanol (8 mL/mmol). Cyclohexene and $Pd(OH)_2/C$ were added and refluxed at 100 °C until TLC indicated completion. The reaction mixture was allowed to cool to room temperature and filtered through celite. The crude material was purified with column chromatography.

General Procedure D for the Synthesis of Compounds 5, 6 and 9. Acetyl protected compounds **3**, **4** and **8** were dissolved in methanol (20 mL/mmol). Potassium carbonate was added and stirred vigorously for 3 h. The solvent was removed *in vacuo* and the crude material was purified with column chromatography.

6-*N*-(1-Adamantyl)-5'-*O*-acetyladenosine (3). **3** was synthesised according to the general procedure A, using chloride **2** (0.2 g, 0.48 mmol), amantadine hydrochloride (0.14 g, 0.72 mmol) and DIPEA (0.84 mL, 4.80 mmol). After purification with column chromatography (methanol/DCM, 2–6%) monoacetylated **3** was obtained as a white solid as the major product (0.13 g, 62% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.31 (1H, s, adenine H), 8.22 (1H, s, adenine H), 6.61 (1H, s, NH), 5.89 (1H, d, *J* 5.1, 1'-H), 5.56 (1H, d, *J* 5.7, 2'-OH), 5.37 (1H, d, *J* 5.4, 3'-OH), 4.67 (1H, dd, *J* 10.5, 5.2, 2'-H), 4.32 (1H, dd, *J* 11.8, 3.7, 5'-H*H*), 4.26 (1H, dd, *J* 10.5, 5.4, 3'-H), 4.16 (1H, dd, *J* 11.8, 6.1, 5'-*H*H), 4.07 (1H, m, 4'-H), 2.21 (6H, m, 6 x adamantyl H), 2.08 (3H, m, 3 x adamantyl H), 2.00 (3H, s, CH_3), 1.68 (6H, m, 6 x adamantyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.1, 154.4, 151.9, 148.4, 139.5, 119.9, 87.9, 81.5, 72.8, 70.3, 63.9, 52.1, 41.0, 36.0, 29.0, 20.6; HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{30}N_5O_5$ $[MH]^+$ 444.2241, found 444.2229; purity UPLC-MS 99%, retention time = 2.92 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)-5'-*O*-acetyladenosine (4). 4 was synthesised according to the general procedure A, using chloride **2** (0.20 g, 0.48 mmol), (1*R*,2*R*)-1-amino-2-

benzyloxycyclopentane (0.13 mL, 0.72 mmol) and triethylamine (0.19 mL, 1.34 mmol). After purification with column chromatography (methanol/DCM, 1–4%) monoacetylated product **4** was obtained as a pale yellow solid as the major product (0.12 g, 52% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.34 (1H, s, adenine H), 8.25 (1H, br s, adenine H), 7.90 (1H, m, NH), 7.33-7.18 (5H, m, 5 x phenyl H), 5.93 (1H, d, *J* 5.1, 1'-H), 5.57 (1H, d, *J* 5.7, 2'-OH), 5.37 (1H, d, *J* 5.3, 3'-OH), 4.68 (1H, dd, *J* 10.4, 5.1, 2'-H), 4.63-4.51 (3H, m, CH_2Ph and 1-H), 4.33 (1H, dd, *J* 11.8, 3.6, 5'-*H*H), 4.27 (1H, dd, *J* 10.4, 5.3, 3'-H), 4.18 (1H, dd, *J* 11.8, 6.2, 5'-H*H*), 4.09 (1H, m, 4'-H), 4.01 (1H, m, 2-H), 2.13-1.90 (5H, m, 2 x cyclopentyl H and CH_3), 1.80-1.55 (4H, m, 4 x cyclopentyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.1, 154.2, 152.5, 139.4, 138.9, 128.1, 127.3, 127.1, 114.5, 87.8, 84.1, 81.5, 72.9, 70.3, 70.1, 63.9, 56.4, 30.2, 21.4, 20.6; HRMS (ESI) calcd for $C_{24}H_{30}O_5N_6$ $[MH]^+$ 484.2191, found 484.2172; purity UPLC-MS 99%, retention time = 2.55 min.

6-*N*-(1-Adamantyl)adenosine (5).[48] **5** was synthesised according to the general procedure D, using **3** (0.03 g, 0.06 mmol) and potassium carbonate (0.005 g, 0.04 mmol). After purification with column chromatography (methanol/DCM, 4%) product **5** was obtained as a white solid (0.03 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.35 (1H, s, adenine H), 8.21 (1H, s, adenine H), 6.65 (1H, s, NH), 5.87 (1H, d, *J* 6.1, 1'-H), 5.44 (1H, d, *J* 6.1, 2'-OH), 5.37 (1H, dd, *J* 7.1, 4.6, 5'-OH), 5.19 (1H, d, *J* 4.6, 3'-OH), 4.62 (1H, dd, *J* 11.1, 6.1, 2'-H), 4.15 (1H, m, 3'-H), 3.97 (1H, m, 4'-H), 3.69 (1H, m, 5'-H*H*), 3.55 (1H, m, 5'*H*H), 2.23 (6H, m, 6 x adamantyl H), 2.10 (3H, m, 3 x adamantyl H), 1.69 (6H, m, 6 x adamantyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.5, 151.6, 148.2, 139.6, 120.1, 88.0, 85.8, 73.4, 70.6, 61.6, 52.2, 41.0, 36.0, 29.0; HRMS (ESI^+) *m/z* calcd for $C_{20}H_{28}N_5O_4$ $[MH]^+$ 402.2136, found 402.2137; purity UPLC 99%, retention time = 2.66 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)adenosine (6).[49] **6** was synthesised according to the general procedure D, using **4** (0.01 g, 0.02 mmol) and potassium carbonate (0.01 g, 0.07 mmol). After purification with column chromatography (methanol/DCM, 2–3%) product **6** was obtained as a white solid (0.01 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.37 (1H, s, adenine H), 8.24 (1H, br s, adenine H), 7.94 (1H, m, NH), 7.33-7.19 (5H, m, 5 x phenyl H), 5.89 (1H, d, *J* 6.1, 1'-H), 5.46-5.37 (2H, m, 2'-OH and

5'-OH), 5.19 (1H, d, *J* 4.6, 3'-OH), 4.68-4.50 (4H, m, 1-H, 2'-H and C*H*$_2$Ph), 4.15 (1H, dd, *J* 7.8, 4.6, 3'-H), 4.05-3.94 (2H, m, 2-H and 4'-H), 3.68 (1H, dt, *J* 12.0, 4.1, 5'-*H*H), 3.56 (1H, m, 5'-H*H*), 2.14-1.89 (2H, m, 2 x cyclopentyl H), 1.77-1.56 (4H, m, 4 x cyclopentyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.2, 152.2, 139.6, 138.9, 128.1, 127.3, 127.1, 87.9, 85.8, 84.1, 73.5, 70.6, 70.1, 61.6, 30.1, 21.4; HRMS (ESI) calcd for $C_{22}H_{28}O_5N_5$ $[MH]^+$ 442.2085, found 442.2097; purity UPLC-MS 98%, retention time = 2.30 min.

6-*N*-((1*R*,2*R*)-2-(Hydroxy)cyclopentyl)adenosine (7).[28] **7** was synthesised according to the general procedure C, using **4** (0.03 g, 0.06 mmol), cyclohexene (0.25 mL, 2.43 mmol) and Pd(OH)$_2$/C (20 wt. %, 0.01 g). After purification with column chromatography (methanol/DCM, 2–8%) product **7** was obtained as a white solid (0.02 g, 95% yield). Minor quantities of the monoacetylated product were also isolated. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.37 (1H, s, adenine H), 8.20 (1H, br s, adenine H), 7.74 (1H, d, *J* 6.8, NH), 5.89 (1H, d, *J* 6.1, 1'-H), 5.48-5.36 (2H, m, 2'-OH and 5'-OH), 5.19 (1H, d, *J* 4.6, 3'-OH), 4.87 (1H, m, 2-OH), 4.62 (1H, app. dd, *J* 11.3, 6.1, 2'-H), 4.29 (1H, br s, 1-H), 4.15 (1H, m, 3'-H), 4.06 (1H, m, 2-H), 3.97 (1H, m, 4'-H), 3.68 (1H, dt, *J* 11.9, 3.9, 5'-*H*H), 3.55 (1H, m, 5'-H*H*), 2.07 (1H, m, 1 x cyclopentyl H), 1.88 (1H, m, 1 x cyclopentyl H), 1.74-1.59 (2H, m, 2 x cyclopentyl H), 1.59-1.45 (2H, m, 2 x cyclopentyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.7, 152.2, 139.6, 87.9, 85.9, 76.0, 73.5, 70.6, 61.6, 58.8, 32.3; HRMS (ESI) calcd for $C_{15}H_{22}O_5N_5$ $[MH]^+$ 352.1615, found 352.1616; purity UPLC-MS 99%, retention time = 1.46 min.

Tri-*O*-acetyl-6-*N*-[(3-*endo*)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]adenosine (8). 8 was synthesised according to the general procedure A, using chloride **2** (0.3 g, 0.73 mmol), (3-*endo*)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine (0.45 g, 2.91 mmol) and DIPEA (0.15 mL, 0.88 mmol). After purification with column chromatography (methanol/DCM, 5–10%) product **8** was obtained as a white solid (0.21 g, 54% yield). ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.30 (1H, br s, adenine H), 8.25 (1H, s, adenine H), 6.24 (1H, d, *J* 5.3, 1'-H), 6.03 (1H, t, *J* 5.3, 2'-H), 5.73 (1H, dd, *J* 5.3, 4.8, 3'-H), 4.83 (1H, br s, 3-H), 4.49-4.37 (3H, m, 4'-H and 5'-H$_2$), 3.44-3.42 (2H, m, 1- and 5-H), 2.77 (3H, s, NCH$_3$), 2.70-2.61 (2H, m, 2 x granatyl H), 2.20-2.03 (12H, m, 3 x CH$_3$ and 3 x granatyl H), 1.77-1.61 (3H, m, 3 x granatyl H), 1.43-1.40

(2H, m, 2 x granatyl H); ^{13}C NMR (100 MHz, MeOD-*d*$_4$) δ 172.2, 171.4, 171.2, 155.6, 154.3, 140.9, 87.9, 81.6, 74.3, 72.0, 64.2, 54.0, 41.7, 40.0, 32.6, 25.7, 20.6, 20.4, 20.2, 14.0; HRMS (ESI$^+$) *m/z* calcd for $C_{25}H_{35}N_6O_7$ 531.2562 $[MH]^+$, found 531.2553; purity UPLC-MS 96%, retention time = 2.14 min.

6-*N*-[(3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]non-3-yl]adenosine (9). **9** was synthesised according to the general procedure D, using **8** (0.05 g, 0.10 mmol) and potassium carbonate (0.005 g, 0.03 mmol). The crude product was dissolved in acetone and passed through filtered paper to give **9** as a white solid (0.04 g, 99% yield). ^{1}H NMR (300 MHz, MeOH-*d*$_4$) δ 8.24 (1H, s, adenine H), 8.21 (1H, br s, adenine H), 5.95 (1H, d, *J* 6.5, 1'-H), 4.77 (1H, br s overlapping, 3-H), 4.74 (1H, dd, *J* 6.4, 5.2, 2'-H), 4.32 (1H, dd, *J* 5.1, 2.5, 3'-H), 4.17 (1H, app q, *J* 2.4, 4'-H), 3.89 (1H, dd, *J* 12.6, 2.4, 5'-*H*H), 3.74 (1H, dd, *J* 12.6, 2.6, 5'-H*H*), 3.13-3.10 (2H, m, 1- and 5-H), 2.60-2.50 (5H, m, NCH_3 and 2 x granatyl H), 2.16-1.98 (3H, m, 3 x granatyl H), 1.58-1.45 (3H, m, 3 x granatyl H), 1.16-1.12 (2H, m, 2 x granatyl H); ^{13}C NMR (100 MHz, MeOH-*d*$_4$) δ 153.7, 141.4, 128.5, 91.4, 88.3, 75.5, 72.7, 63.6, 52.7, 42.7, 40.8, 33.4, 25.8, 15.0; HRMS (ESI) calcd for $C_{19}H_{29}O_4N_6$ $[MH]^+$ 405.2245, found 405.2243; purity UPLC-MS 99%, retention time = 1.41 min.

6-*N*-(1-Adamantyl)-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (11). 11 was synthesised according to the general procedure A, using chloride **10** (0.3 g, 0.82 mmol), amantadine hydrochloride (0.46 g, 2.45 mmol) and DIPEA (2.61 mL, 15.01 mmol). After purification with column chromatography (methanol/DCM, 2%) product **11** was obtained as a white solid (0.28 g, 72% yield). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 8.27 (1H, s, adenine H), 8.17 (1H, s, adenine H), 7.51 (1H, t, *J* 5.6, amide NH), 6.62 (1H, s, amine NH), 6.34 (1H, d, *J* 1.5, 1'-H), 5.41 (1H, dd, *J* 6.1, 1.5, 2'-H), 5.38 (1H, dd, *J* 6.1, 1.8, 3'-H), 4.53 (1H, d, *J* 1.8, 4'-H), 2.80 (2H, m, *CH*$_2$CH$_3$), 2.21 (6H, m, 6 x adamantyl H), 2.09 (3H, m, 3 x adamantyl H), 1.68 (6H, m, 6 x adamantyl H), 1.54 (3H, s, CH_3), 1.35 (3H, s, CH_3), 0.62 (3H, t, *J* 7.2, CH_2*CH*$_3$); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 168.1, 154.4, 151.8, 148.0, 139.9, 119.6, 112.9, 89.5, 85.8, 83.2, 83.0, 52.1, 41.0, 36.0, 33.0, 29.0, 26.7, 25.0, 13.8; HRMS (ESI$^+$) *m/z* calcd for $C_{25}H_{35}N_6O_4$ $[MH]^+$ 483.2714, found 483.2718.

6-*N*-(3-Hydroxy-1-adamantyl)-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (12). 12 was synthesised according to the general procedure A, using chloride **10** (0.07 g, 0.19 mmol), 3-amino-1-adamantanol (0.05 g, 0.29 mmol) and triethylamine (0.5 mL, 3.6 mmol). After purification with column chromatography (methanol/DCM, 2–5%) product **12** was obtained as a white solid (0.07 g, 78% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.26 (1H, s, adenine H), 8.16 (1H, s, adenine H), 7.49 (1H, t, *J* 5.6, amide NH), 6.74 (1H, s, amine NH), 6.33 (1H, d, *J* 1.1, 1'-H), 5.43-5.35 (2H, m, 2'-H and 3'-H), 4.53 (2H, s, 4'-H and OH), 2.81 (2H, m, CH_2CH$_3$), 2.18-2.01 (8H, m, 8 x adamantyl H), 1.65-1.43 (9H, m, 6 x adamantyl H and CH$_3$), 1.34 (3H, s, CH$_3$), 0.62 (3H, t, *J* 7.2, CH$_2$$CH_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.1, 154.3, 151.7, 148.1, 140.0, 119.6, 112.9, 89.5, 85.6, 83.2, 83.0, 67.5, 54.6, 48.9, 44.2, 34.9, 33.0, 30.1, 26.7, 25.0, 13.8; HRMS (ESI$^+$) *m/z* calcd for $C_{25}H_{35}N_6O_5$ 499.2663 $[MH]^+$, found 499.2650; purity UPLC-MS 99%, retention time = 2.62 min.

6-*N*-(2-Adamantyl)-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (13). 13 was synthesised according to the general procedure A, using chloride **10** (0.02 g, 0.06 mmol), 2-adamantylamine hydrochloride (0.03 g, 0.18 mmol) and DIPEA (0.05 mL, 0.27 mmol). After purification with column chromatography (methanol/DCM, 2%) product **13** was obtained as a white solid (0.02 g, 69% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.29 (1H, s, adenine H), 8.17 (1H, br s, adenine H), 7.48 (1H, t, *J* 5.7, amide NH), 7.05 (1H, br s, NH), 6.34 (1H, s, 1'-H), 5.45-5.36 (2H, m, 2'- and 3'-H), 4.53 (1H, s, 4'-H), 4.36 (1H, br s, adamantyl H), 2.80 (2H, m, CH_2CH$_3$), 2.11-2.05 (4H, m, 4 x adamantyl H), 1.84 (6H, m, 6 x adamantyl H), 1.72 (2H, m, 2 x adamantyl H), 1.53-1.50 (5H, m, 2 x adamantyl H and CH$_3$), 1.34 (3H, s, CH$_3$), 0.59 (3H, t, *J* 7.2, CH$_2$$CH_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.1, 154.1, 152.3, 140.3, 112.9, 89.5, 85.9, 83.2, 83.1, 37.1, 36.9, 36.8, 33.0, 30.9, 26.7, 26.6, 25.0, 13.8; HRMS (ESI) calculated for $C_{25}H_{35}N_6O_4$ $[MH]^+$ 483.2714, found 483.2707; purity UPLC-MS 94%, retention time = 3.12 min.

***6*-*N*-[(3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]non-3-yl]-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (14). 14** was synthesised according to the general procedure A, using chloride **10** (0.1 g, 0.27 mmol), (3-*endo*)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine (0.17 g, 1.09 mmol) and

DIPEA (0.06 mL, 0.33 mmol). After purification with column chromatography (methanol/DCM, 5–10%) product **14** was obtained as a white solid (0.11 g, 85% yield). ^{1}H NMR (300 MHz, MeOD-d_4) δ 8.21 (2H, m, 2 x adenine H), 6.35 (1H, d, *J* 1.0, 1'-H), 5.63 (1H, dd, *J* 6.1, 1.8, 3'-H), 5.51 (1H, m, 2'-H), 4.77 (1H, br s, 3-H), 4.64 (1H, d, *J* 1.7, 4'-H), 3.27-3.23 (1H, m, 1- and 5-H), 2.85 (2H, m, CH_2CH_3), 2.67-2.50 (5H, m, NCH_3 and 2 x granatyl H), 2.20-2.00 (3H, m, 3 x granatyl H), 1.65-1.51 (6H, m, CH_3 and 3 x granatyl H), 1.42 (3H, s, CH_3) 1.34-1.19 (2H, m, 2 x granatyl H), 0.63 (3H, t, *J* 7.3, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) 171.6, 155.6, 154.1, 142.0, 114.9, 92.4, 88.7, 85.3, 85.2, 53.3, 42.2, 40.4, 34.7, 33.1, 27.1, 25.8, 25.3, 14.6, 14.0; HRMS calculated for $C_{24}H_{36}O_4N_7$ $[MH]^+$ 486.2823, found 486.2810; purity UPLC-MS 99%, retention time = 1.97 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (15). 15 was synthesised according to the general procedure A, using chloride **10** (0.2 g, 0.54 mmol), (1*R*,2*R*)-1-amino-2-benzyloxycyclopentane (0.15 mL, 0.82 mmol) and triethylamine (0.21 mL, 1.51 mmol). Following removal of the solvent from the reaction mixture, the residue was dissolved in ethyl acetate (100 mL) and washed with water (2 x 50 mL). The organic phase was then dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. After purification with column chromatography (methanol/DCM, 1–3%) product **15** was obtained as a pale yellow solid (0.22 g, 79% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.27 (1H, s, adenine H), 8.20 (1H, br s, adenine H), 7.91 (1H, m, amine NH), 7.51 (1H, t, *J* 5.7, amide NH), 7.33-7.20 (5H, m, 5 x phenyl H), 6.34 (1H, s, 1'-H), 5.43-5.36 (2H, m, 2'- and 3'-H), 4.66-4.49 (4H, m, 4'-H, CH_2Ph and 1-H), 4.01 (1H, m, 2-H), 2.81 (2H, m, CH_2CH_3), 2.12-1.89 (2H, m, 2 x cyclopentyl H), 1.80-1.56 (4H, m, 4 x cyclopentyl H), 1.54 (3H, s, CH_3), 1.34 (3H, s, CH_3), 0.61 (3H, t, *J* 7.2, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.1, 154.1, 152.4, 148.2, 139.9, 138.9, 128.1, 127.3, 127.1, 112.9, 89.5, 85.8, 84.0, 83.2, 83.1, 70.1, 56.4, 33.0, 30.2, 26.7, 25.0, 21.3, 13.8; HRMS (ESI) calculated for $C_{27}H_{35}O_5N_6$ $[MH]^+$ 523.2663, found 523.2649; purity UPLC-MS 99%, retention time = 3.08 min.

6-*N*-(1-Adamantyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (16). 16 was synthesised according to the general procedure B, using **11** (0.10 g, 0.21 mmol), acetic acid (10 mL) and water (3 mL). After purification with column chromatography (methanol/DCM, 2–6%) product **16** was obtained as a white solid (0.09 g, 96% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.89 (1H, t, *J* 5.5, amide NH), 8.39 (1H, s, adenine H), 8.27 (1H, s, adenine H), 6.77 (1H, s, amine NH), 5.95 (1H, d, *J* 7.7, 1'-H), 5.75 (1H, d, *J* 3.8, 3'-OH), 5.56 (1H, d, *J* 6.2, 2'-OH), 4.61 (1H, m, 2'-H), 4.30 (1H, d, *J* 1.2, 4'-H), 4.14 (1H, m, 3'-H), 3.22 (2H, m, CH_2CH_3), 2.23 (6H, m, 6 x adamantyl H), 2.09 (3H, m, 3 x adamantyl H), 1.68 (6H, m, 6 x adamantyl H), 1.08 (3H, t, *J* 7.2, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1, 154.6, 151.6, 148.0, 140.4, 120.4, 87.9, 84.7, 73.1, 71.9, 52.2, 41.0, 36.0, 33.2, 29.0, 14.7; IR [cm^{-1}] 3218, 2905, 2847, 1644; HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{31}N_6O_4$ $[MH]^+$ 443.2401, found 443.2393; purity UPLC 99%, retention time = 3.06 min.

6-*N*-(3-Hydroxy-1-adamantyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (17). 17 was synthesised according to the general procedure B, using **12** (0.01 g, 0.02 mmol), acetic acid (3.6 mL) and water (1.2 mL). After purification with column chromatography (methanol/DCM, 1–5%) product **17** was obtained as a white solid (0.007 g, 78% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.88 (1H, t, *J* 5.5, amide NH), 8.40 (1H, s, adenine H), 8.27 (1H, s, adenine H), 6.89 (1H, s, amine NH), 5.95 (1H, d, *J* 7.6, 1'-H), 5.74 (1H, d, *J* 4.2, 3'-OH), 5.57 (1H, d, *J* 6.4, 2'-OH), 4.61 (1H, m, 2'-H), 4.55 (1H, s, adamantyl OH), 4.30 (1H, d, *J* 1.2, 4'-H), 4.14 (1H, m, 3'-H), 3.21 (2H, m, CH_2CH_3), 2.20-2.05 (8H, m, 8 x adamantyl H), 1.66-1.43 (6H, m, 6 x adamantyl H), 1.08 (3H, t, *J* 7.2, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1, 154.5, 151.6, 148.0, 140.4, 120.3, 87.8, 84.7, 73.1, 72.0, 67.5, 54.7, 48.8, 44.2, 34.9, 33.3, 30.1, 14.7; HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{31}N_6O_5$ $[MH]^+$ 459.2350, found 459.2346; purity UPLC 94%, retention time = 2.18 min.

6-*N*-(2-Adamantyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (18). 18 was synthesised according to the general procedure B, using **13** (0.02 g, 0.04 mmol), acetic acid (1.6 mL) and water (0.4 mL). After purification with column chromatography (methanol/DCM, 3–10%) product **18** was obtained

as a white solid (0.02 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.86 (1H, m, amide NH), 8.43 (1H, s, adenine H), 8.27 (1H, s, adenine H), 7.15 (1H, br s, amine NH), 5.97 (1H, d, *J* 7.5, 1'-H), 5.74 (1H, d, *J* 4.3, 3'-OH), 5.54 (1H, d, *J* 6.4, 2'-OH), 4.62 (1H, m, 2'-H), 4.37 (1H, br s, adamantyl H), 4.30 (1H, d, *J* 1.4, 4'-H), 4.14 (1H, m, 3'-H), 3.21 (2H, m, CH_2CH_3), 2.13-2.08 (4H, m, 4 x adamantyl H), 1.85 (6H, m, 6 x adamantyl H), 1.73 (2H, m, 2 x adamantyl H), 1.56-1.52 (2H, m, 2 x adamantyl H), 1.08 (3H, t, *J* 7.2, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1, 154.3, 152.3, 140.6, 114.5, 87.8, 84.6, 73.1, 71.9, 37.2, 36.9, 33.3, 30.9, 26.8, 14.7; HRMS (ESI) calcd for $C_{22}H_{31}N_6O_4$ $[MH]^+$ 443.2401, found 443.2392; purity UPLC-MS 99%, retention time = 2.69 min.

***6*-*N*-[(3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]non-3-yl]-5'-ethylamino-5'-oxo-5'-deoxyadenosine (19). 19** was synthesised according to the general procedure B, using **14** (0.03 g, 0.06 mmol), acetic acid (4.8 mL) and water (1.2 mL). After purification with column chromatography (methanol/DCM, 5–10%, with an additional 1% aqueous ammonia) product **19** was obtained as a white solid (0.03 g, 99% yield). ^{1}H NMR (300 MHz, MeOD-d_4) δ 8.30 (1H, br s, adenine H), 8.27 (1H, s, adenine H), 6.01 (1H, d, *J* 7.7, 1'-H), 4.81 (1H partially behind solvent signal, m, 3-H), 4.75 (1H, dd, *J* 7.6, 4.8, 2'-H), 4.47 (1H, d, *J* 1.5, 4'-H), 4.31 (1H, dd, *J* 4.8, 1.4, 3'-H), 3.37 (2H, m, CH_2CH_3), 3.28 (2H partially behind solvent signal, m, 1- and 5-H), 2.69-2.56 (5H, m, NCH_3 and 2 x granatyl H), 2.21-2.04 (3H, m, 3 x granatyl H), 1.68-1.56 (3H, m, 3 x granatyl H), 1.30 (2H, m, 2 x granatyl H), 1.21 (3H, t, *J* 7.3, CH_2CH_3); ^{13}C NMR (100 MHz, MeOD-d_4) δ 172.1, 155.8, 153.9, 142.1, 90.5, 86.5, 75.0, 73.4, 53.5, 42.1, 40.2, 35.1, 33.0, 25.7, 15.0, 14.4; HRMS (ESI) calcd for $C_{21}H_{32}N_7O_4$ $[MH]^+$ 446.2510, found 446.2523; purity UPLC-MS 99%, retention time = 1.62 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (20). 20 was synthesised according to the general procedure B, using **15** (0.01 g, 0.02 mmol), acetic acid (1.6 mL) and water (0.4 mL). After purification with column chromatography (methanol/DCM, 2–4%) product **20** was obtained as a white solid (0.01 g, 99% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.90 (1H, t, *J* 5.6, amide NH), 8.41 (1H, s, adenine H), 8.30 (1H, br s, adenine H), 8.03 (1H, m, amine NH), 7.33-7.20 (5H,

m, 5 x phenyl H), 5.98 (1H, d, *J* 7.6, 1'-H), 5.73 (1H, d, *J* 4.3, 3'-OH), 5.53 (1H, d, *J* 6.5, 2'-OH), 4.67-4.53 (4H, m, 2'-H, C*H*$_2$Ph and 1-H), 4.32 (1H, d, *J* 1.5, 4'-H), 4.15 (1H, m, 3'-H), 4.03 (1H, m, 2-H), 3.23 (2H, m, *CH*$_2$CH$_3$), 2.08 (1H, m, 1 x cyclopentyl H), 1.96 (1H, m, 1 x cyclopentyl H), 1.79-1.60 (4H, m, 4 x cyclopentyl H), 1.09 (3H, t, *J* 7.2, CH$_2$*CH*$_3$); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 169.1, 154.3, 152.3, 140.4, 138.9, 128.1, 127.3, 127.1, 120.1, 87.8, 84.7, 84.1, 73.1, 72.0, 70.1, 56.4, 33.2, 30.2, 21.4, 14.7; HRMS (ESI) calcd for $C_{24}H_{31}O_5N_6$ $[MH]^+$ 483.2350, found 483.2339; purity UPLC-MS 98%, retention time = 2.59 min.

6-*N*-((1*R*,2*R*)-2-(Hydroxy)cyclopentyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (21). 21 was synthesised according to the general procedure C, using **20** (0.08 g, 0.17 mmol), cyclohexene (0.66 mL, 6.55 mmol) and Pd(OH)$_2$/C (20 wt. %, 0.02 g). After purification with column chromatography (methanol/DCM, 2–8%) product **21** was obtained as a white solid (0.07 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 8.91 (1H, t, *J* 5.4 Hz, amide NH), 8.41 (1H, s, adenine H), 8.26 (1H, br s, adenine H), 7.84 (1H, m, amine NH), 5.97 (1H, d, *J* 7.6, 1'-H), 5.75 (1H, d, *J* 4.3, 3'-OH), 5.55 (1H, d, *J* 6.5, 2'-OH), 4.86 (1H, m, 2-OH), 4.62 (1H, m, 2'-H), 4.31 (2H, m, 1-H and 4'-H), 4.14 (1H, m, 3'-H), 4.06 (1H, m, 2-H), 3.23 (2H, m, *CH*$_2$CH$_3$), 2.10 (1H, m, 1 x cyclopentyl H), 1.90 (1H, m, 1 x cyclopentyl H), 1.74-1.42 (4H, m, 4 x cyclopentyl H), 1.09 (3H, t, *J* 7.2, CH$_2$*CH*$_3$); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 169.1, 154.8, 152.2, 140.3, 87.8, 84.6, 76.0, 73.1, 72.0, 58.8, 33.3, 32.3, 20.4, 14.7; HRMS (ESI) calcd for $C_{17}H_{25}O_5N_6$ $[MH]^+$ 393.1881, found 393.1873; purity UPLC-MS 99%, retention time = 2.21 min.

***6*-*N*-[(3-*endo*)-3-*tert*-Butyloxycarbonylamino-9-azabicyclo[3.3.1]non-3-yl]-5'-ethylamino-2',3'-*O*-isopropylidene-5'-oxo-5'-deoxyadenosine (23). 23** was synthesised according to the general procedure A, using chloride **10** (0.03 g, 0.08 mmol), *tert*-butyl-9-azabicyclo[3.3.1]nonan-3-ylcarbamate (**22**) (0.04 g, 0.16 mmol) and triethylamine (0.41 mL, 2.94 mmol). Following removal of the solvent from the reaction mixture, the residue was dissolved in ethyl acetate (100 mL) and washed with water (2 x 50 mL). The organic phase was then dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. After purification with column chromatography (methanol/DCM, 1–2%) product **23** was obtained as a pale

yellow solid (0.03 g, 67% yield). ^{1}H NMR (300 MHz, MeOD-d_4) δ 8.18 (1H, s, adenine H), 8.13 (1H, m, adenine H), 6.33 (1H, s, 1'-H), 6.16 (1H, m, 1- or 5-H), 5.61 (1H, m, 2'-H), 5.49 (1H, m, 3'-H), 5.42 (1H, m, 1- or 5-H), 4.62 (1H, d, *J* 1.7, 4'-H), 3.29 (1H, m, granatyl H), 2.85 (2H, m, CH_2CH_3), 2.42-2.16 (3H, m, 3 x granatyl H), 1.79-1.34 (22H, m, 2 x CH_3, -$C(CH_3)_3$ and 7 x granatyl H), 0.66 (3H, dt, *J* 14.7, 7.2, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) 171.8, 155.4, 153.7, 140.6, 117.1, 115.0, 93.0, 88.9, 85.5, 85.3, 45.1, 44.1, 35.0, 33.6, 33.2, 32.5, 31.8, 30.9, 28.9, 27.2, 25.5, 15.0, 14.3; HRMS calculated for $C_{28}H_{42}O_6N_7$ $[MH]^+$ 572.3191, found 572.3187.

***6-N*-[(3-*endo*)-3-Amino-9-azabicyclo[3.3.1]non-3-yl]-5'-ethylamino-5'-oxo-5'-deoxyadenosine (24). 24** was synthesised according to the general procedure B, using **23** (0.02 g, 0.03 mmol), acetic acid (1.5 mL) and water (0.5 mL). The crude product was purified by prep-LC, with an eluent gradient of 100% A to 60% D in 40 minutes. The fractions were collected and dried by lyophilization and product **24** was obtained as a white solid as the TFA salt (0.03 g, 99% yield). ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.34 (1H, s, adenine H), 8.28 (1H, s, adenine H), 6.35 (1H, br s, 1- or 5-H), 6.03 (1H, d, *J* 7.6, 1'-H), 5.59 (1H, br s, 1- or 5-H), 4.74 (1H, dd, *J* 7.5, 4.8, 2'-H), 4.48 (1H, d, *J* 1.6, 4'-H), 4.30 (1H, dd, *J* 4.8, 1.5, 3'-H), 3.36 (2H, q, *J* 7.3, CH_2CH_3), 3.00 (1H, ddd, *J* 18.3, 12.2, 5.8, 3-H), 2.57 (2H, m, 2 x granatyl H), 2.18 (1H, m, 1 x granatyl H), 1.87-1.64 (7H, m, 7 x granatyl H), 1.20 (3H, t, *J* 7.3, CH_2CH_3); ^{13}C NMR (100 MHz, MeOD-d_4) δ 172.2, 155.2, 152.6, 150.7, 141.1, 121.8, 90.6, 86.4, 75.0, 73.5, 45.1, 35.1, 31.0, 15.0, 14.7; ^{19}F NMR (376 MHz, DMSO-d_6) δ -77.3; HRMS (ESI) calcd for $C_{20}H_{30}O_4N_7$ $[MH]^+$ 432.2354, found 432.2343; purity UPLC-MS 99%, retention time = 1.80 min.

***6-N*-[(3-*endo*)-3-Aminodimethyl-9-azabicyclo[3.3.1]non-3-yl]-5'-ethylamino-5'-oxo-5'-deoxyadenosine (25).** Formic acid (0.02 mL) and formaldehyde (37% aq. solution, 0.04 mL) were added to **24** (0.01 g, 0.02 mmol) and stirred at 90 °C overnight. Additional formic acid (0.02 mL) and formaldehyde (37% aq. solution, 0.04 mL) were added and stirred at 105 °C for 3 h. The reaction mixture was allowed to cool to room temperature and made alkaline with 1M NaOH solution. This was then thoroughly extracted with ethyl acetate (3 x 100 mL) and the combined organic extracts were washed with

sat. aq. $NaHCO_3$ (50 mL), water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* to give **25** as white solid (0.006 g, 67% yield). ^{1}H NMR (300 MHz, MeOH-d_4) δ 8.27 (1H, s, adenine H), 8.21 (1H, s, adenine H), 6.26 (1H, br s, 1- or 5-H), 6.01 (1H, d, *J* 7.6, 1'-H), 5.49 (1H, br s, 1- or 5-H), 4.78 (1H, dd, *J* 7.7, 4.8, 2'-H), 4.46 (1H, d, *J* 1.4, 4'-H), 4.30 (1H, dd, *J* 4.8, 1.4, 3'-H), 3.38 (2H, m, CH_2CH_3), 2.45 (9H, m, 2 x CH_3 and 3 x granatyl H), 2.18 (1H, m, 1 x granatyl H), 1.77-1.58 (7H, m, 7 x granatyl H), 1.20 (3H, t, *J* 7.3, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1, 155.1, 153.3, 151.5, 140.8, 121.6, 90.4, 86.4, 75.0, 73.2, 58.6, 41.8, 35.1, 30.8, 15.1, 14.9; HRMS (ESI) calcd for $C_{22}H_{34}O_4N_7$ $[MH]^+$ 460.2667, found 460.2661; purity UPLC-MS 99%, retention time = 1.87 min.

6-*N*-(1-Adamantyl)-5'-(2-fluorophenylthio)-2',3'-*O*-isopropylidene-5'-deoxyadenosine (30). **30** was synthesised according to the general procedure A, using chloride **29** (0.1 g, 0.23 mmol), amantadine hydrochloride (0.13 g, 0.69 mmol) and DIPEA (0.18 mL, 1.03 mmol). After purification with column chromatography (methanol/DCM, 0.5%) product **30** was obtained as a white solid (0.09 g, 69% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.29 (1H, s, adenine H), 8.23 (1H, s, adenine H), 7.38 (1H, td, *J* 7.8, 1.6, Ar H), 7.31-7.07 (3H, m, 3 x Ar H), 6.67 (1H, s, amine NH), 6.17 (1H, d, *J* 2.2, 1'-H), 5.51 (1H, dd, *J* 6.2, 2.2, 2'-H), 5.05 (1H, dd, *J* 6.2, 2.7, 3'-H), 4.20 (1H, td, *J* 6.9, 2.5, 4'-H), 3.25 (2H overlapping with solvent signal, m, 5'-H_2), 2.22 (6H, m, 6 x adamantyl H), 2.09 (3H, m, 3 x adamantyl H), 1.75-1.63 (6H, m, 6 x adamantyl H), 1.49 (3H, s, CH_3), 1.31 (3H, s, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.3, 158.9, 154.4, 151.9, 147.7, 139.8, 131.1, 128.5 (d, *J* 7.9), 124.9 (d, *J* 3.6), 121.8 (d, *J* 17.1), 119.9, 115.6 (d, *J* 21.9), 113.2, 89.5, 85.1, 83.4, 83.2, 52.2, 41.0, 36.0, 34.4, 29.0, 26.8, 25.1; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.4; HRMS (ESI^+) *m/z* calcd for $C_{29}H_{35}N_5O_3FS$ $[MH]^+$ 552.2439, found 552.2425.

***6*-*N*-[(3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]non-3-yl]-5'-(2-fluorophenylthio)-2',3'-*O*-isopropylidene-5'-deoxyadenosine (31).** **31** was synthesised according to the general procedure A, using chloride **29** (0.1 g, 0.23 mmol), (3-*endo*)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine (0.14 g, 0.92 mmol) and DIPEA (0.04 mL, 0.28 mmol). After purification with column chromatography (methanol/DCM, 5–10%) product **31** was obtained as a pale yellow solid (0.08 g, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ

8.33 (1H, s, adenine H), 7.76 (1H, s, adenine H), 7.36 (1H, m, Ar H), 7.20 (1H, m, Ar H), 7.04-6.97 (2H, m, 2 x Ar H), 6.01 (1H, d, *J* 2.0, 1'-H), 5.54 (1H, dd, *J* 6.3, 1.9, 2'-H), 5.52 (1H overlapping with 2'-H signal, br s, NH), 5.10 (1H, dd, *J* 6.3, 2.8, 3'-H), 4.75 (1H, br s, 3-H), 4.37 (1H, td, *J* 7.2, 2.8, 4'-H), 3.25 (H, dd, *J* 13.7, 7.6, 5-*H*H), 3.17 (1H, dd, *J* 13.7, 6.5, 5-H*H*), 3.11 (2H, m, 1- and 5-H), 2.65-2.52 (2H, m, 2 x granatyl H), 2.50 (3H, s, NCH_3), 2.04-1.91 (3H, m, 3 x granatyl H), 1.56 (3H, s, CH_3), 1.53 (1H, m, granatyl H), 1.37 (3H, s, CH_3), 1.35-1.29 (2H, m, 2 x granatyl H), 1.02 (2H, m, 2 x granatyl H); ^{13}C NMR (75 MHz, $CDCl_3$) 163.5, 160.2, 154.6, 153.5, 139.3, 133.3, 129.2 (d, *J* 8.0), 124.5 (d, *J* 3.8), 122.0 (d, *J* 17.5), 115.9 (d, *J* 22.5), 114.3, 91.3, 86.6, 84.2, 84.1, 51.4, 40.3, 36.2, 33.6, 27.1, 25.4, 24.2, 14.4; ^{19}F NMR (376 MHz, DMSO-d_6) δ -108.4; HRMS calculated for $C_{28}H_{36}O_3N_6FS$ $[MH]^+$ 555.2548, found 555.2548; purity UPLC-MS 93%, retention time = 2.88 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)-5'-(2-fluorophenylthio)-2',3'-*O*-isopropylidene-5'-deoxyadenosine (32). 32 was synthesised according to the general procedure A, using chloride **29** (0.05 g, 0.11 mmol), (1*R*,2*R*)-1-amino-2-benzyloxycyclopentane (0.03 mL, 0.17 mmol) and triethylamine (0.04 mL, 0.31 mmol). After purification with column chromatography (methanol/DCM, 2%) product **32** was obtained as a sticky yellow oil (0.06 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.32 (1H, s, adenine H), 8.26 (1H, br s, adenine H), 7.96 (1H, m, amine NH), 7.39 (1H, td, *J* 7.8, 1.6, Ar H), 7.32-7.06 (8H, m, 8 x Ar H), 6.19 (1H, d, *J* 2.1, 1'-H), 5.51 (1H, dd, *J* 6.2, 2.1, 2'-H), 5.07 (1H, dd, *J* 6.2, 2.6, 3'-H), 4.67-4.47 (3H, m, 1-H and CH_2Ph), 4.21 (1H, td, *J* 7.1, 2.6, 4'-H), 4.01 (1H, m, 2-H), 3.25 (2H overlapping with solvent signal, m, 5'-H_2), 2.13-1.89 (2H, m, 2 x cyclopentyl H), 1.79-1.57 (4H, m, 4 x cyclopentyl H), 1.49 (3H, s, CH_3), 1.31 (3H, s, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.3, 158.9, 154.2, 152.5, 147.9, 139.8, 138.9, 131.1, 128.5 (d, *J* 7.9), 128.1, 127.3, 127.1, 124.9 (d, *J* 3.5), 121.8 (d, *J* 17.1), 115.6 (d, *J* 22.0), 113.2, 89.4, 85.2, 84.0, 83.4, 83.2, 70.1, 56.5, 34.4, 30.1, 26.8, 25.1, 21.4; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.4; HRMS (ESI) calculated for $C_{31}H_{35}O_4N_5FS$ $[MH]^+$ 592.2388, found 592.2378; purity UPLC-MS 94%, retention time = 3.98 min.

6-*N*-((1*R*,2*R*)-2-(Hydroxy)cyclopentyl)-5'-(2-fluorophenylthio)-2',3'-*O*-isopropylidene-5'-deoxyadenosine (33). 33 was synthesised according to the general procedure A using chloride **29** (0.05 g, 0.11 mmol), (1*R*,2*R*)-2-aminocyclopentanol hydrochloride (0.02 g, 0.14 mmol) and triethylamine (0.04 mL, 0.31 mmol). After purification with column chromatography (methanol/DCM, 2%) product **33** was obtained as a white solid (0.04 g, 73% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.31 (1H, s, adenine H), 8.22 (1H, br s, adenine H), 7.76 (1H, m, NH), 7.39 (1H, td, *J* 7.8, 1.6, Ar H), 7.31-7.07 (3H, m, 3 x Ar H), 6.18 (1H, d, *J* 2.1, 1'-H), 5.51 (1H, dd, *J* 6.2, 2.1, 2'-H), 5.06 (1H, dd, *J* 6.2, 2.7, 3'-H), 4.86 (1H, m, 2-OH), 4.27 (1H, br s, 1-H), 4.21 (1H, td, *J* 7.1, 2.6, 4'-H), 4.06 (1H, m, 2-H), 3.26 (2H overlapping with solvent signal, m, 5'-H_2), 2.06 (1H, m, 1 x cyclopentyl H), 1.89 (1H, m, 1 x cyclopentyl H), 1.72-1.60 (2H, m, 2 x cyclopentyl H), 1.60-1.43 (5H, m, 2 x cyclopentyl H and CH_3), 1.32 (3H, s, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.3, 158.9, 154.7, 152.5, 139.8, 131.1, 128.6 (d, *J* 7.9), 124.9 (d, *J* 3.5), 121.8 (d, *J* 17.1), 115.6 (d, *J* 22.0), 113.2, 89.5, 85.1, 83.4, 83.2, 76.0, 58.9, 34.4, 32.3, 26.8, 25.1, 20.4; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.4; HRMS (ESI) calculated for $C_{24}H_{29}O_4N_5FS$ $[MH]^+$ 502.1919, found 502.1912; purity UPLC-MS 99%, retention time = 3.14 min.

6-*N*-(1-Adamantyl)-5'-(2-fluorophenylthio)-5'-deoxyadenosine (34). 34 was synthesised according to the general procedure B, using **30** (0.01 g, 0.02 mmol), acetic acid (3.2 mL) and water (0.8 mL). After purification with column chromatography (methanol/DCM, 1–3%) product **34** was obtained as a white solid (0.004 g, 40% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (1H, s, adenine H), 8.21 (1H, s, adenine H), 7.46 (1H, td, *J* 7.8, 1.6, Ar H), 7.29-7.12 (3H, m, 3 x Ar H), 6.60 (1H, s, NH), 5.87 (1H, d, *J* 5.7, 1'-H), 5.51 (1H, d, *J* 6.1, 2'-OH), 5.37 (1H, d, *J* 5.0, 3'-OH), 4.82 (1H, dd, *J* 11.1, 5.7, 2'-H), 4.22 (1H, dd, *J* 8.7, 4.9, 3'-H), 4.00 (1H, m, 4'-H), 3.42 (1H, dd, *J* 13.8, 5.5, 5'-*H*H), 3.31 (1H overlapping with solvent signal, m, 5'-H*H*), 2.23 (6H, m, 6 x adamantyl H), 2.10 (3H, m, 3 x adamantyl H), 1.68 (6H, m, 6 x adamantyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.1, 158.7, 154.4, 151.9, 148.5, 139.7, 130.5, 128.1 (d, *J* 7.9), 125.0 (d, *J* 3.4), 122.6 (d, *J* 17.1), 119.9, 115.5 (d, *J* 21.8), 87.7, 82.8, 72.7, 72.5, 52.1, 41.0, 36.0,

34.6, 29.0; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.78; HRMS (ESI$^+$) *m/z* calcd for $C_{26}H_{31}N_5O_3FS$ $[MH]^+$ 512.2126, found 512.2130.

6-*N*-[(3-*endo*)-9-Methyl-9-azabicyclo[3.3.1]non-3-yl]-5'-(2-fluorophenylthio)-5'-deoxyadenosine (35). 35 was synthesised according to the general procedure B, using **31** (0.01 g, 0.02 mmol), acetic acid (3.2 mL) and water (0.8 mL). After purification with column chromatography (methanol/DCM, 1–10%, with an additional 1% aqueous ammonia) product **35** was obtained as a white solid (0.005 g, 50% yield). ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.24 (1H, s, adenine H), 8.17 (1H, s, adenine H), 7.45 (1H, m, Ar H), 7.21 (1H, m, Ar H), 7.07-7.01 (2H, m, 2 x Ar H), 5.95 (1H, d, *J* 5.2, 1'-H), 4.84 (1H, 2'-H under solvent signal as determined by COSY analysis), 4.83 (1H, br s, 3-H, partially hidden by solvent signal), 4.37 (1H, m, 3'-H), 4.20 (1H, m, 4'-H), 3.40 (2H, m, CH_2CH_3), 3.31 (2H, 1- and 5-H under solvent signal as determined by COSY analysis), 2.70-2.57 (5H, m, NCH_3 and 2 x granatyl H), 2.16-2.04 (3H, m, 3 x granatyl H), 1.69-1.59 (3H, m, 3 x granatyl H), 1.32-1.29 (2H, m, 2 x granatyl H); ^{13}C NMR (100 MHz, MeOD-d_4) δ 163.8, 155.6, 154.0, 140.9, 133.2, 129.7 (d, *J* 7.9), 125.7 (d, *J* 3.7), 116.5 (d, *J* 22.5), 90.1, 85.0, 74.7, 74.1, 53.7, 40.2, 36.5, 32.9, 25.7, 14.3, 7.6; ^{19}F NMR (376 MHz, DMSO-d_6) δ -111.5; HRMS (ESI$^+$) *m/z* calcd for $C_{25}H_{32}N_6O_3FS$ $[MH]^+$ 515.2235, found 515.2240; purity UPLC-MS 99%, retention time = 2.24 min.

6-*N*-((1*R*,2*R*)-2-(Benzyloxy)cyclopentyl)-5'-(2-fluorophenylthio)-5'-deoxyadenosine (36).[47] **36** was synthesised according to the general procedure B, using **32** (0.02 g, 0.03 mmol), acetic acid (3.2 mL) and water (0.8 mL). After purification with column chromatography (methanol/DCM, 1–3%) product **36** was obtained as a pale yellow solid (0.02 g, 99% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.35 (1H, s, adenine H), 8.24 (1H, br s, adenine H), 7.89 (1H, m, amine NH), 7.46 (1H, m, Ar H), 7.33-7.10 (8H, m, 8 x Ar H), 5.90 (1H, d, *J* 5.6, 1'-H), 5.52 (1H, d, *J* 6.0, 2'-OH), 5.38 (1H, d, *J* 5.1, 3'-OH), 4.81 (1H, dd, *J* 11.1, 5.6, 2'-H), 4.68-4.50 (3H, m, 1-H and CH_2Ph), 4.21 (1H, dd, *J* 8.7, 4.8, 3'-H), 4.00-3.97 (2H, m, 4'-H and 2-H), 3.42 (1H, dd, *J* 13.8, 5.5, 5'-*H*H), 3.32 (1H overlapping with solvent signal, m, 5'-H*H*) 2.14-1.91 (2H, m, 2 x cyclopentyl H), 1.77-1.58 (4H, m, 4 x cyclopentyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ

161.1, 158.7, 154.2, 152.5, 139.6, 139.0, 130.5, 128.1, 128.0, 127.3, 127.1, 125.0 (d, *J* 3.4), 122.6 (d, *J* 17.2), 155.5 (d, *J* 21.9), 87.6, 84.1, 82.8, 72.7, 72.6, 70.1, 56.4, 34.6, 30.2, 21.4; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.8; HRMS (ESI) calcd for $C_{28}H_{31}O_4N_5FS$ $[MH]^+$ 552.2075, found 552.2071; purity UPLC-MS 99%, retention time = 3.14 min.

6-*N*-((1*R*,2*R*)-2-(Hydroxy)cyclopentyl)-5'-(2-fluorophenylthio)-5'-deoxyadenosine (37).[14] **37** was synthesised according to the general procedure B, using **33** (0.01 g, 0.02 mmol), acetic acid (3.2 mL) and water (0.8 mL). After purification with column chromatography (methanol/DCM, 1–5%) product **37** was obtained as a white solid (0.005 g, 56% yield). The *O*-acetylated product was also isolated in small quantities. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.34 (1H, s, adenine H), 8.21 (1H, br s, adenine H), 7.69 (1H, d, *J* 7.1, amine NH), 7.47 (1H, td, *J* 7.8, 1.6, Ar H), 7.31-7.10 (3H, m, 3 x Ar H), 5.89 (1H, d, *J* 5.7, 1'-H), 5.54 (1H, d, *J* 6.0, 2'-OH), 5.40 (1H, d, *J* 5.0, 3'-OH), 4.87 (1H, d, *J* 4.0, 2-OH), 4.80 (1H, dd, *J* 10.9, 5.6, 2'-H), 4.30 (1H, br s, 1-H), 4.21 (1H, dd, *J* 8.6, 4.7, 3'-H), 4.09-3.95 (2H, m, 4'-H and 2-H), 3.42 (1H, dd, *J* 13.7, 5.5, 5'-*H*H), 3.32 (1H, m, 5'-H*H*), 2.07 (1H, m, 1 x cyclopentyl H), 1.90 (1H, m, 1 x cyclopentyl H), 1.72-1.42 (4H, m, 4 x cyclopentyl H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.1, 158.7, 154.6, 152.4, 139.6, 130.5 (d, *J* 1.7), 128.1 (d, *J* 7.9), 125.0 (d, *J* 3.4), 122.6 (d, *J* 17.1), 115.5 (d, *J* 21.8), 87.6, 82.8, 76.1, 72.7, 72.6, 58.8, 34.6, 32.3, 20.4; ^{19}F NMR (376 MHz, DMSO-d_6) δ -110.8; HRMS (ESI) calcd for $C_{21}H_{25}O_4N_5FS$ $[MH]^+$ 462.1606, found 462.1590; purity UPLC-MS 95%, retention time = 2.35 min.

Biology Materials. Yeast extract and yeast nitrogen base were purchased from Difco (Franklin Lakes, NJ). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Constructs and DNA Manipulation. p426-GPD-A_1R was kindly provided by Professor Arthur Christopoulos and Dr. Lauren May (Monash University, Australia). Mammalian expression vectors containing the A_1R, A_{2A}R, A_{2B}R and A_3R were purchased from Missouri S&T cDNA Resource Center (http://cdna.org) (Rolla, MO). DNA manipulations were performed using standard techniques. Oligonucleotides were supplied by Invitrogen and PCR amplification performed using FastStart Taq

polymerase (Roche Diagnostics, Burgess Hill, UK). All constructs generated by PCR were sequenced by GATC (GATC Biotech, London, UK) prior to use.

General Yeast Methods. General yeast procedures were performed as described previously.[16] Cells were routinely cultured in YPDA (yeast, peptone, dextrose and adenine). Yeast transformations were achieved using the lithium acetate/single-stranded DNA/polyethylene glycol method as previously described.[50] Cells were selected for uracil biosynthesis and routinely cultured in synthetic dropout media lacking uracil (SD-URA).

Yeast Strain Construction. The production of the dual reporter strains expressing chimeras of five C-terminal amino acids of human Gα protein with the yeast Gpa1p, 1-467 (GPA1/Gα) has been described previously.[16] Mammalian GPCRs were introduced into the yeast strains (MMY12, MMY14, MMY19, MMY22, MMY23, MMY25 and MMY28) using the p426-GPD expression plasmid. Positive isolates were selected upon their ability to generate β-galactosidase activity above basal when stimulated with 100 μM NECA. For chimeric strains that did not initially appear to functionally couple ($n \geq 16$ isolates) to the ARs, expression and membrane localization were confirmed using fluorescence microscopy.

Yeast Reporter Gene Assay. Yeast cells were treated with compounds as described in Dowell and Brown.[16] Initially cells were cultured overnight in SD-URA at 30°C. Cells were diluted 1:10 in SD-URA and allowed to grow for 8 hours at 30°C. Finally cell density was adjusted to an OD_{600} of 0.02 and treated with 1% (v/v) of the appropriate compound dissolved in DMSO in a 96-well plate for 16 hours at 30°C. For compounds dissolved in other solvents the media was supplemented with 1% (v/v) DMSO prior to treatment. To compensate for an elevated basal signal, the $A_{2A}R$ was routinely cultured in SD-URA lacking histidine (SD-URA-HIS) and treatment media supplemented with 5mM 3-amino-triazole. All strains used in this study contain the *lacZ* gene under the control of the pheromone-responsive *FUS1* promoter. To assess β-galactosidase activity cells were lysed as previously described.[19,51-53] 2-Nitrophenyl β-D-galactopyranoside (ONPG) was used as a chromogenic substrate for β-galactosidase and detected by OD_{430}. Absorbance was measured using a Mithras LB940 microplate reader (Berthold Technologies, Harpenden, UK). The strains are Δ*far1* and are therefore incapable of cell cycle arrest induced by the pheromone-

response. Consequently, these cells grow throughout treatment. To compensate for variability in cell number and bleed through from the chromogenic reporter, cell density was measured by OD_{620} and a response calculated as $(OD_{430}-OD_{620})/OD_{620}$.

Confocal Microscopy of Yeast. To visual receptor expression C-terminal in-frame fusion constructs between the A_1R, $A_{2A}R$ and $A_{2B}R$ and GFP was generated using the two-step cloning method described by Ladds *et al.*[54] These receptors were expressed in yeast using p426-GPD vector consistent with their un-tagged counterparts. Isolates were cultured for 24 h in SD-URA. 100 μL cells were harvested by centrifugation, washed in PBS and briefly sonicated. Cells were imaged using a True Confocal Scanner Leica TCS SP5 microscope (Leica Microsystems Ltd., Milton Keynes, UK) and were processed using ImageJ as described previously.[55]

Mammalian Cell Culture and Transfection. CHO-K1 cells, provided by Dr Ewan St. John Smith (University of Cambridge), were routinely cultured in Hams-F12, supplemented with 10% fetal bovine serum (FBS), and maintained at 37°C, in humidified air with 5% CO_2. Cells were transfected with 2 μg DNA using FuGene® 6 at a 3:1 (w:v) DNA:FuGene 6 ratio. Cells were harvested 48 h post transfection for assaying.

cAMP Accumulation Assay. Transfected cells were washed with PBS and resuspended in stimulation buffer (PBS containing 0.1% BSA and 25 μM rolipram). Cells were seeded at 2500 cells per well in 384-well white Optiplates. Cells were then simultaneously incubated with 10 μM forskolin (to stimulate cAMP production) and adenosine receptors ligands (ranging between 1 μM to 10 pM) for 30 min at room temperature. Cells were then lysed and the extent of cAMP accumulation measured using a LANCE® cAMP Detection Kit (PerkinElmer). Plates were read using a Mithras LB 940 multimode plate reader (Berthold Technologies).

Data Analysis. Data were analyzed using Prism 6.0e (Graphpad Software, San Diego, CA). Concentration response curves were fitted using the three-parameter logistic equation to obtain EC_{50} and E_{max}. Schild analysis was performed in Prism as described by Motulsky and Christopoulos.[56] Non-linear regression of the operational model of pharmacological agonism[36] was used to obtain values for efficacy

(log τ) and the equilibrium dissociation constant (log K_A). These values were then used to quantify signaling bias as the change in log (τ/K_A) relative to NECA.[37] We have used this method previously to enable quantification of G protein bias[19,20] but here we have extended the analysis to include receptor selectivity. Since the receptors are expressed in the same cell background, and that NECA is a full potent agonist against all receptor subtypes, we reasoned that changes in log (τ/K_A) for a given ligand, relative to NECA for each AR would provide a quantitative means of comparing receptor selectivity. Statistically significant differences were detected using one-way ANOVA with Bonferroni's or Dunnett's multiple comparison tests or Student T-tests as appropriate and a probability (p) < 0.05 was considered significant.

Homology Modeling and Docking. The protein sequence of the human A_1R (accession number P30542) was aligned with an agonist (UK-432097)-bound human $A_{2A}R$ template (PDB ID: 3QAK) using PSI-Coffee[57] (Figure S4). MODELLER v9.14[58] was used to build 500 models and the best model selected according to the inbuilt molecular probability function. The ligands were constructed *ab initio* in Chem3D Pro v14.0 (PerkinElmer, Waltham, MA) and energy-minimised using the included MM2 force field. For each ligand a library of 200 conformers was generated using OMEGA v2.5 (OpenEye Scientific Software, Santa Fe, NM). FRED RECEPTOR v2.2.5 (OpenEye Scientific Software) was utilized to generate a docking template whereas the binding site was defined as a box of V = 9486 $Å^3$ around the bound UK-432097 agonist. For predicting the binding poses, the ligands were docked into this binding site template using FRED v2.1 (OpenEye Scientific Software) that utilizes an exhaustive process to position and score all conformers of a ligand at all possible positions within the defined binding site. Binding poses that did not form a hydrogen bond with either Thr-$91^{3.36}$, Asn-$254^{6.55}$ or Thr-$277^{7.42}$ were discarded ($h$$A_1R$ numbering according to P30542, superscript: Ballesteros-Weinstein numbering[45]). Ten docking poses were generated for each ligand, ranked using the inbuilt Chemgauss3 scoring function and visualized with PyMOL v1.7 (Schrödinger LLC, Portland, OR).

Supporting Information

Synthesis procedures and spectral data for synthetic intermediates, reproduction of ^{1}H and ^{13}C NMR spectra, purity assessment for final compounds, Schild analysis of compounds **36** and **37**, functional assessment of A_3R in yeast and predicted docking poses for compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes:

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Abbreviations Used

AR, adenosine receptor; A_1R, A_1 adenosine receptor; $A_{2A}R$, A_{2A} adenosine receptor; $A_{2B}R$, A_{2B} adenosine receptor; A_3R, A_3 adenosine receptor; CCPA, 2-chloro-N^6-cyclopentyladenosine; DIPEA, di-*iso*-propyl ethyl amine (Hünig's base); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gpa1p, guanine nucleotide-binding protein alpha-1 subunit; NECA, 5'-*N*-ethylcarboxamidoadenosine.

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